SATB2, a novel p73-interacting protein: characterization and role in osteosarcoma

by

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Institute of Medical Science
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Abstract

p73 shares many structural and functional similarities with its homologue, the p53 tumour suppressor. Many p73 protein isoforms result from both alternative splicing and differential promoter utilization. Transcriptionally active (TA) isoforms are pro-apoptotic, while N-terminally truncated (∆N) variants are anti-apoptotic and act as dominant-negative inhibitors of TAp73 and p53. p73 has been shown to activate the transcription of genes involved in cell cycle arrest and apoptosis in response to DNA damaging agents, including chemotherapies. We hypothesize that the activity of p73, like many transcription factors, may be modulated by binding to regulatory proteins. A novel p73-binding protein that we have identified is special AT-rich sequence binding protein 2 (SATB2), which is a nuclear matrix attachment region (MAR)-binding protein. It has important functions in the regulation of gene transcription, neuronal and osteoblast (OB) differentiation, and craniofacial patterning. The SATB2 family member, SATB1, has been implicated in breast tumour growth and metastasis. Here, I describe the initial characterization of the p73-SATB2 interaction. SATB2 binds to and stabilizes TAp73α, leading to the enhancement of TAp73α-mediated induction of p53-upregulated modulator of apoptosis (PUMA; apoptotic gene) and p21 (cell cycle gene). Conversely, SATB2
does not bind to but decreases TAp73β levels, resulting in inhibition of TAp73β-mediated transcription. Thus, our findings reveal a role for SATB2 in the regulation of p73 stability and function. Moreover, we demonstrate that SATB2 is overexpressed in osteosarcoma (OS), while it is undetectable in other sarcomas, thereby suggesting that SATB2 may be a potential biomarker that can distinguish OS from other sarcomas. Loss of SATB2 also inhibits the migration and invasion capabilities of OS cells, and suggests that SATB2 promotes both migration and invasion in OS. Therefore, I present work showing that SATB2 is a novel p73-binding partner that differentially regulates the stability and function of the C-terminal isoforms of p73, as well as the novel involvement of SATB2 in OS invasion.
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Abbreviations

ATCC: American Type Culture Collection
ATF4: activating transcription factor 4
ATM: ataxia telangiectasia mutated
BAX: B-cell lymphoma 2-associated X protein
BRMS1: breast cancer metastasis suppressor 1
Bsp: bone sialoprotein
ChIP: chromatin immunoprecipitation
CHX: cycloheximide
CBP: CREB-binding protein
CT: computed tomography
DBD: DNA-binding domain
df: degrees of freedom
DMEM: Dulbecco’s modified Eagle’s medium
ECM: extracellular matrix
ERBB2: v-erb-b2 erythroblastic leukemia viral oncogene homolog 2
ES: embryonic stem
FBS: fetal bovine serum
HA: hemagglutinin
HEK: human embryonic kidney

HNSCC: head and neck squamous cell carcinoma

HOM: homeodomain

Hoxa2: homeobox A2

IB: immunoblot

IHC: immunohistochemistry

IP: immunoprecipitation

LSAMP: limbic system-associated membrane protein

MAR: matrix attachment region

MDM2: mouse double minute 2

MMP: matrix metalloproteinase

MOI: multiplicity of infection

MRI: magnetic resonance imaging

MSTS: Musculoskeletal Tumor Society

NF2: neurofibromatosis-2

NGF: nerve growth factor

NQO1: NAD(P)H:quinone oxidoreductase-1

OB: osteoblast

Ocn: osteocalcin

OD: oligomerization domain

OS: osteosarcoma
PBS: phosphate-buffered saline

PDZ: postsynaptic density-95 (PSD-95), discs-large (Dlg), zonula occludens-1 (ZO-1)

PEI: polyethylenimine

PET: positron emission tomography

PI3K: phosphatidylinositol-3-kinase

PIAS1: protein inhibitor of activated STAT 1

PUMA: p53-upregulated modulator of apoptosis

qRT-PCR: quantitative reverse transcription-polymerase chain reaction

RB1: retinoblastoma

RNAi: RNA interference

ROBO4: roundabout 4

RTS: Rothmund-Thomson syndrome

RT-PCR: reverse transcription-polymerase chain reaction

RUNX2: runt-related transcription factor 2

SAM: sterile α motif

SATB2: special AT-rich sequence binding protein 2

SDS-PAGE: sodium dodecyl sulphate-polyacrylamide gel electrophoresis

shRNA: short hairpin RNA

TAD: transactivation domain

TBS: Tris-buffered saline

TBST: Tris-buffered saline with Tween 20
TGFB1: transforming growth factor beta 1

TID: transactivation inhibitory domain

TIMP: tissue inhibitor of metalloproteinases

uPA: urokinase plasminogen activator

VEGF: vascular endothelial growth factor

VEGFR: vascular endothelial growth factor receptor

YAP1: Yes-associated protein 1
Chapter 1: 
General introduction to p73, SATB2 and osteosarcoma

1.1. p73: a member of the p53 family

Since its discovery more than 30 years ago, p53 has emerged as a focus of intense study for its role as a tumour suppressor gene. p53 is mutated in approximately 50% of human cancers and inactivated in many others (Vogelstein et al., 2000). p73 and p63 were identified as p53 family members in the late 1990s (Kaghad et al., 1997; Yang et al., 1998), and although they all share many structural and functional similarities, several differences exist among them.

1.1.1. Structure and functional effects

The structure of p73 (Figure 1.1) is much like that of the other p53 family members, p53 and p63. The p53 family proteins have three common functional domains: an N-terminal transactivation domain (TAD), a central DNA-binding domain (DBD) and a C-terminal oligomerization domain (OD). In general, p73 and p63 isoforms that contain the TAD are denoted by TAp73 or TAp63, while those that are derived from the internal cryptic promoter and lack this N-terminal TAD are denoted by ΔNp73 or ΔNp63. Moreover, the α forms of p73 and p63 contain a sterile α motif (SAM) domain downstream of the OD. Downstream of this SAM domain, p63 also has a transactivation inhibitory domain (TID) and is regulated by an intramolecular mechanism whereby the TID binds to and inhibits the transactivating ability of the TAD (Serber et al., 2002). Therefore, p63 isoforms lacking this TID have a greater transactivation potential. Similar to p63, the SAM domain and extreme C-terminus of p73 are...
able to suppress its transcriptional activity, but these regions do not bind to the TAD of p73 (Liu and Chen, 2005).

All three family members have isoforms that are derived from both alternative splicing and differential promoter utilization (reviewed in Murray-Zmijewski et al., 2006). p73, in particular, has many isoforms generated at both the N- and C-terminus. Five N-terminal variants have been identified: TAp73, the full-length form; ΔNp73, generated from an alternative promoter within intron 3; ΔNp73’, generated from an alternative translation initiation site due to an upstream premature stop; and ΔEx2p73 and ΔEx2/3p73, generated from alternative splicing of exons 2 and 3 (Yang et al., 2000; Fillippovich et al., 2001; Ishimoto et al., 2002; Zaika et al., 2002). The N-terminally truncated isoforms are collectively called ΔTAp73. A number of C-terminal isoforms have also been described, including α, β, γ, δ, ε, ζ and η (De Laurenzi et al., 1998; De Laurenzi et al., 1999; Ishimoto et al., 2002). The p73 gene, with different combinations of the N- and C-termini, can theoretically generate at least 35 transcripts that encode 28 different proteins.
Figure 1.1. Schematic diagram of the p73 structure. (A) The gene structure of p73 consists of 14 exons (numbered) and 2 transcriptional start sites, P1 and P2 (indicated by arrows). P2 is the internal cryptic promoter located within intron 3 (designated by exon 3’). Untranslated regions are shaded in black and the exon regions encoding the functional domains of p73 are designated by colour: TAD in pink, DBD in blue, OD in green, and SAM in purple. p73 isoforms are generated by both alternative splicing and differential promoter utilization. The five N-terminal variants are: TAp73, the full-length form generated from P1; ∆Np73, generated from P2 (orange); ∆Np73’, generated from an alternative translation initiation site due to an upstream premature stop; and ∆Ex2p73 and ∆Ex2/3p73, generated from alternative splicing of exons 2 and 3. Seven C-terminal isoforms are shown: α, β, γ, δ, ε, ζ and η. p73η is generated by an alternative translation termination site within exon 13. (B) The protein structure of p73 shows the functional domains—TAD, DBD, OD and SAM—of TAp73α (the full-length isoform), TAp73β (which lacks the SAM domain), and the N-terminally truncated variants ∆Np73α (which lacks the TAD) and ∆Np73β (which lacks both the TAD and SAM domain). Only the α and β C-terminal isoforms are shown.
The TA forms of p53 family members can all activate the transcription of genes involved in cell cycle arrest and apoptosis in response to DNA-damaging agents, including chemotherapeutic drugs. Both p73 and p63 can bind to p53 DNA-consensus-binding sites and activate numerous p53-target genes (reviewed in Harms et al., 2004). p53 family members form homotetramers in order to bind to these DNA sites. ΔNp73 inhibits full-length p53 and TAp73 by two mechanisms: 1) ΔNp73 can sequester p53 and TAp73 by forming transcriptionally inactive heterotetramers, and 2) ΔNp73 can form homotetramers and compete with active p53 and TAp73 homotetramers for DNA-binding sites (Kartasheva et al., 2002; Nakagawa et al., 2002; Stiewe et al., 2002; Zaika et al., 2002). ΔNp73 is induced by p53 and TAp73 such that a dominant-negative feedback loop is generated (Grob et al., 2001; Kartasheva et al., 2002; Nakagawa et al., 2002; Vossio et al., 2002). Furthermore, ΔNp73 can also inhibit p53 by preventing p53 activation (Wilhelm et al., 2010). In addition, some p53 “gain of function” mutants can bind to TAp73 (and p63), thereby inhibiting its pro-apoptotic abilities (Di Como et al., 1999; Marin et al., 2000; Strano et al., 2000; Gaiddon et al., 2001). Only the TA forms of p73 and p63 can induce apoptosis, which may account for their tumour-suppressor-like abilities (pro-apoptotic). The ΔN forms act as dominant-negative inhibitors and function like oncogenes (anti-apoptotic). Therefore, the TA:ΔN ratio plays a crucial role in tumourigenesis.

Since the TA and ΔN isoforms of p73 have important and opposing roles in cancer development, the majority of p73 studies focus on the differences between these N-terminal isoforms. In contrast, the functional differences among the various p73 C-terminal isoforms are not well studied. The two most commonly detected p73 C-terminal splice variants are p73α and β. TAp73β is more potent at activating the transcription of many target genes and inducing apoptosis than TAp73α (Jost et al., 1997; Zhu et al., 1998; Di Como et al., 1999; Lee and La
79. Thangue, 1999). This difference is most likely due to the ability of the SAM domain and extreme C-terminus of TAp73α to suppress its transcriptional activity since artificial truncation mutants of TAp73α lacking this region possess activity similar to TAp73β in transactivation assays (Liu and Chen, 2005). One possibility is that the SAM domain recruits as-yet unidentified regulatory proteins.

1.1.2. Pathway regulators and effectors

Protein-protein interactions and post-translational modifications, such as phosphorylation, acetylation, ubiquitination and sumoylation, affect the activity, stability and subcellular localization of p53. Likewise, the p73 pathway is complex and involves numerous regulators and effectors, and the best described are shown in Figure 1.2. p73 activity is initiated by DNA damage resulting in phosphorylation of the protein kinase ataxia telangiectasia mutated (ATM; Bakkenist and Kastan, 2003), and subsequent phosphorylation of both the non-receptor tyrosine kinase c-Abl (Baskaran et al., 1997; Shafman et al., 1997) and E2F1, a regulator of cell cycle progression and cell death (Urist et al., 2004). p73 is phosphorylated by c-Abl (Agami et al., 1999; Gong et al., 1999; Yuan et al., 1999) and then acetylated by the acetyltransferase p300/CREB-binding protein (CBP; Costanzo et al., 2002), thereby enhancing the transcriptional activity of p73. The regions of p73 to which these proteins bind are shown in Figure 1.3. E2F1 can also activate p73 transcription by binding to the promoter of p73 itself (Irwin et al., 2000). Once TAp73 is induced, it can transactivate the promoters of many p53-target genes, thereby inducing cell cycle arrest or apoptosis (Fontemaggi et al., 2002; Harms et al., 2004). Such target genes include the cell cycle inhibitor p21, and the pro-apoptotic genes p53-upregulated modulator of apoptosis (PUMA) and B-cell lymphoma 2-associated X protein (BAX). TAp73
transcriptionally activates *PUMA* and *BAX*, and PUMA mediates the conformational change and mitochondrial translocation of BAX, which leads to cytochrome C release and subsequent apoptosis (Melino et al., 2004).
Figure 1.2. **DNA damage activation of p73.** DNA damage activates the upstream regulators of p73 (ATM, c-Abl and E2F-1). Once activated, TAp73 transactivates the promoters of its downstream effectors (including \( p21 \), \( PUMA \) and \( BAX \)). \( p21 \) induction leads to cell cycle arrest; PUMA causes the conformational change and mitochondrial translocation of BAX, which leads to cytochrome C release and subsequent apoptosis.
Figure 1.3. Regulatory proteins bind to different regions of p73. A subset of identified binding partners for p73 (and the putative binding regions) are shown. These include proteins that regulate p73 levels and/or activity.
The stability and degradation of p73 are regulated by several protein-protein interactions. The regions of p73 to which a subset of these proteins bind are shown in Figure 1.3. Only a subset of these regulatory proteins binds to p73 in an isoform-specific manner (e.g., TA versus ΔN, or α versus β). In contrast to p53, p73 is not degraded by mouse double minute 2 (MDM2). Instead, MDM2 binding to p73 results in stabilization of p73 and inhibition of its transcriptional activity (Balint et al., 1999; Dobbelstein et al., 1999; Zeng et al., 1999). Ubiquitin-dependent proteasomal degradation of p73 is regulated by proteins other than MDM2. ITCH promotes the ubiquitination and subsequent proteasomal degradation of p73α and β, as well as p63 (Rossi et al., 2005; Rossi et al., 2006). In contrast, Yes-associated protein 1 (YAP1) binds to p73 and prevents proteasomal degradation by ITCH, thereby prolonging the half-life of p73 (Levy et al., 2007). NEDL2 also promotes the ubiquitination of p73α and β, but this interaction unexpectedly results in the increased stability and transcriptional activity of TAp73 (Miyazaki et al., 2003).

p73 is also regulated by ubiquitin-independent mechanisms that involve the proteasome. NAD(P)H:quinone oxidoreductase-1 (NQO1) binds to p73α (but not β) and p53, and prevents them from undergoing proteasomal degradation in an ubiquitin-independent manner (Asher et al., 2002). UFD2a binds to and decreases the half-life of p73α, but not p73β or p53, leading to the inhibition of the transcriptional and pro-apoptotic activity of p73α (Hosoda et al., 2005). UFD2a was also shown to regulate proteasomal turnover of p73α in an ubiquitin-independent manner (Hosoda et al., 2005). Lastly, sumoylation is another important mechanism by which p73 stability is regulated. Protein inhibitor of activated STAT 1 (PIAS1) sumoylates p73α and results in the proteasomal degradation and inhibition of the transcriptional activity of p73 (Minty et al., 2000; Munarriz et al., 2004). To date, most identified regulators of p73 have not been shown to bind to and/or regulate p73 in an isoform-specific manner. Therefore, the regulators
and effectors of p73 allow this transcription factor to function in a tightly-controlled manner in the cell. However, most of these studies to date do not shed light on the specific regulation of different N- or C-terminal isoforms, or why some cells only express a certain subset of isoforms.

1.1.3. Role in development

p73- and p63-deficient mice exhibit prominent developmental defects, indicating the involvement of p73 and p63 in neuronal and epidermal development, respectively (Yang et al., 2000; Yang et al., 1999). The majority of p73-null mice (p73–/–), lacking all of the isoforms of p73, died within two months of birth, but about 25% survived to adulthood (Yang et al., 2000). These mice exhibited hippocampal dysgenesis, hydrocephalus, chronic infections and inflammation, and pheromonal signalling defects (Yang et al., 2000), as well as peripheral sympathetic and cortical neuron loss (Pozniak et al., 2000; Pozniak et al., 2002). Furthermore, these studies revealed that ∆Np73 is the predominant isoform of p73 that is expressed in the developing murine nervous system, and that loss of this anti-apoptotic p73 isoform led to increased apoptosis of sympathetic and cortical neurons. Thus, by opposing the apoptotic function of p53 and TAp63, ∆Np73 acts as a pro-survival protein in developing neurons (Pozniak et al., 2000; Jacobs et al., 2005). In a recent study, ∆Np73-deficient mice (ΔNp73–/–), specifically lacking all variants of ΔNp73, were viable and fertile but exhibited neurodegeneration, and lends further support to a neuroprotective role for ΔNp73 (Wilhelm et al., 2010). Although ΔNp73 is essential for neuronal survival, its overexpression in developing mice has a detrimental developmental effect, resulting in early embryonic lethality (Erster et al., 2006).
While p73 is involved in neuronal development, p63 is a key player in epidermal development. \( p63 \)-deficient mice (\( p63^{−/−} \)), lacking all of the isoforms of p63, had striking limb, craniofacial and epithelial malformations, and died within a few days of birth (Mills et al., 1999; Yang et al., 1999). Other abnormalities in these mice included limb truncations, as well as a complete lack of any epidermis and related structures, such as hair follicles, teeth, mammary and salivary glands. In humans, heterozygous germline \( p63 \) mutations are found in six rare autosomal dominant developmental disorders comprised of various degrees of limb and craniofacial malformations, as well as ectodermal dysplasias (van Bokhoven and McKeon, 2002).

In support of the role of p53 as a tumour suppressor, \( p53 \)-null mice (\( p53^{−/−} \)) develop spontaneous tumours (Donehower et al., 1992). Most \( p53 \)-null mice survive to adulthood (Donehower et al., 1992), and initially, p53 was reported to be dispensable for embryonic development. Upon closer examination, however, it was discovered that some of these mice, mainly female, displayed exencephaly where neural tissue overgrew in the midbrain due to neural tube closure defects (Armstrong et al., 1995; Sah et al., 1995). In humans, germline \( p53 \) mutations have been detected in Li-Fraumeni syndrome, which predisposes these individuals to cancer (Malkin et al., 1990). Based on these knockout mouse models, it is clear that all members of the p53 family have distinct roles not only in development, but in other areas of interest, such as cancer.

### 1.1.4. Role in cancer

In contrast to \( p53 \), \( p73 \) and \( p63 \) are rarely mutated in human cancers. Instead, the transactivation-incompetent isoforms of \( p73 \) and \( p63 \), \( ∆Np73 \) and \( ∆Np63 \), are overexpressed in
various tumours, including brain, thyroid, breast, lung, ovary, prostate, colon, and skin cancer (Wager et al., 2006; Frasca et al., 2003; Dominguez et al., 2005; Massion et al., 2003; Uramoto et al., 2004; Uramoto et al., 2006; Concin et al., 2004; Guan and Chen, 2005; Tuve et al., 2004), as well as in neuroblastoma and head and neck squamous cell carcinoma (HNSCC; Casciano et al., 2002; Sniezek et al., 2004). In addition, the level of ∆Np73 expression has been reported to be an adverse prognostic marker for the majority of the tumour types mentioned above (Wager et al., 2006; Dominguez et al., 2005; Uramoto et al., 2004; Uramoto et al., 2006; Concin et al., 2004; Guan and Chen, 2005; Casciano et al., 2002). In most of these studies, the authors did not distinguish which C-terminal isoform (ie. ∆Np73α or β) was detected. However, in general, most protein expression analyses of tumour cell lines show increased α versus β isoforms (Marin et al., 1998; Irwin et al., 2000; Irwin et al., 2003).

In contrast, TAp73 is induced by a number of chemotherapeutic and DNA-damaging agents, including cisplatin, doxorubicin and gamma irradiation (Agami et al., 1999; Gong et al., 1999; Yuan et al., 1999; Chen et al., 2001; Costanzo et al., 2002; Bergamaschi et al., 2003; Irwin et al., 2003). In cancer cell lines, TAp73 induces apoptosis independent of cellular p53 status, and in certain cases, TAp73β is more effective at inducing apoptosis than p53 or TAp73α (Jost et al., 1997; Kaghad et al., 1997; Ishida et al., 2000). Furthermore, studies of fibroblasts generated from mice with combinations of p53 family member deletions indicated that p53 requires both p73 and p63 in order to induce apoptosis in response to DNA damage (Flores et al., 2002). Therefore, TAp73 is able to induce apoptosis independent of p53, which partly accounts for the efficacy of chemotherapy treatment against tumours with mutated or inactivated p53 (Irwin et al., 2003). Moreover, loss of TAp73 and TAp63 expression has been reported in
leukemias/lymphomas and bladder cancer (Corn et al., 1999; Kawano et al., 1999; Puig et al., 2003; Park et al., 2000).

Initially, the lack of tumours in $p73^{-/-}$ mice (deletion of all p73 isoforms) did not support a role for p73 as a tumour suppressor (Yang et al., 2000). More recent studies involving mice with combinations of p73 and p63 deletions or isoform-specific deletions have proven otherwise. Mice heterozygous for $p73$ and $p63$ mutations ($p73^{+/-};p63^{+/-}$) develop malignant and premalignant lesions at a late age (Flores et al., 2005). This study also provided support for the cooperative roles of p73, p63 and p53 since mice heterozygous for both $p73$ and $p53$ or $p63$ and $p53$ mutations ($p73^{+/-};p53^{+/-}$ or $p63^{+/-};p53^{+/-}$) exhibited a different tumour spectrum, larger tumour burden and more metastasis than mice heterozygous for $p53$ mutations ($p53^{+/-}$). The generation of TAp73- and TAp63-specific knockout mice provided further evidence in support of the role of p73 and p63 as tumour suppressors. TAp73$^{-/-}$ mice, which specifically lack all of the TA (but not ΔN) isoforms of p73, displayed a high incidence of tumours (mostly sarcomas and lymphomas), infertility, hippocampal dysgenesis and genomic instability (Tomasini et al., 2008). Studies of TAp63$^{-/-}$ mice revealed roles for TAp63 in the maintenance of the female germline, senescence, genomic stability, and aging (Suh et al., 2006; Guo et al., 2009; Su et al., 2009). Interestingly, TAp63$^{-/-}$ mice did not develop tumours. Thus, the ratio between the TA and ΔN isoforms of p73 and p63 play a crucial role in tumourigenesis as evident by the interplay between the ΔNp73/ΔNp63 overexpression in tumours and the tumour-suppressor-like ability of TAp73/TAp63. Further studies are needed to help determine the factors or pathways that regulate relative expression of N- and C-terminal isoforms in tumour cells.
1.2. SATB2: a member of the SATB family

Special AT-rich sequence binding protein 2 (SATB2) was identified by our laboratory as a novel p73-interacting protein (see Chapter 2). The SATB family consists of two members: SATB1 and SATB2. SATB2 is the more recently-identified member of this family, and was independently identified in 2003 by two groups—the first group identified SATB2 to be one of the genes disrupted by 2q breakpoints associated with isolated cleft palate (FitzPatrick et al., 2003) and the second group screened for matrix attachment region (MAR)-binding proteins expressed predominantly in pre-B cells (Dobreva et al., 2003).

1.2.1. Structure

Both SATB1 and SATB2 proteins (Figure 1.4) have two CUT domains and one homeodomain. CUT domains are DNA-binding motifs that can bind to DNA independent of or in cooperation with homeodomains (which are often located downstream of a CUT domain). Homeodomains are motifs that bind to DNA and RNA, and often regulate genes involved in differentiation and development. In addition, a “SATB” domain common to both proteins has been identified in the N-terminus (FitzPatrick et al., 2003). This region contains a dimerization domain that is highly homologous to postsynaptic density-95 (PSD-95), discs-large (Dlg), zonula occludens-1 (ZO-1; PDZ) domains (Galande et al., 2001), which may play roles in protein-protein interactions. Both SATB1 and SATB2 also undergo sumoylation by PIAS1 (Gocke et al., 2005; Tan et al., 2008; Dobreva et al., 2003).
Figure 1.4. Schematic diagram of the SATB family protein structure. SATB1 and SATB2 both have a common N-terminal region (SATB) that contains a dimerization domain that is highly homologous to PDZ domains. Both proteins also have three other functional domains: two CUT domains and a homeodomain (HOM). Asterisks indicate lysine sites where sumoylation by PIAS1 occurs.
1.2.2. Function as a transcriptional regulator

The SATB family proteins are highly conserved throughout evolution (FitzPatrick et al., 2003). Like SATB1, SATB2 regulates gene expression either by binding directly to nuclear MARs of target genes, or by binding and altering the transcriptional activity of other transcription factors, acting as a co-activator or co-repressor. MARs are regulatory DNA sequences that are involved in higher-order chromatin organization, which is important in the regulation of gene expression, and in turn, cellular differentiation. For example, SATB2 can bind to MARs in the immunoglobulin μ heavy chain locus in pre-B cells, leading to increased expression (Dobreva et al., 2003). In osteoblast (OB) differentiation, SATB2 regulates bone sialoprotein (Bsp) and homeobox A2 (Hoxa2) by binding to MAR-consensus sequence loci, and also binds to and enhances the transcriptional activity of runt-related transcription factor 2 (RUNX2) and activating transcription factor 4 (ATF4; Dobreva et al., 2006). Therefore, the ability of SATB2 to regulate gene expression translates to crucial roles in development.

1.2.3. Role in development

SATB2 was first identified as a gene essential for craniofacial patterning and disruption of this gene was associated with cleft palate in humans (FitzPatrick et al., 2003). Interestingly, the 2q32-q33 locus, where SATB2 is located, is one of only three genomic regions where haploinsufficiency has been linked with isolated cleft palate (Brewer et al., 1998; Brewer et al., 1999). SATB2 has also been implicated in 2q33.1 microdeletion syndrome, where small deletions of SATB2 are associated with the clinical features, including severe developmental delay, tooth abnormalities, behavioural problems and cleft palate (Rosenfeld et al., 2009). In addition, mice heterozygous for SATB2 mutations (SATB2+/−) displayed phenotypic defects,
including cleft palate, similar to those observed in humans with 2q32 deletions and translocations (Britanova et al., 2006). Loss of SATB2 in mice (SATB2<sup>−/−</sup>) led to perinatal lethality, resulting from multiple craniofacial defects, including cleft palate (Britanova et al., 2006; Dobreva et al., 2006). Thus, SATB2 appears to be an essential regulator of palate development.

As mentioned previously, SATB2 also plays a role in OB differentiation. The developing bones of SATB2<sup>−/−</sup> mice showed decreased bone formation, reduced collagen levels and virtually no osteoid seams (Dobreva et al., 2006). SATB2 regulates several OB-specific genes, such as Bsp and osteocalcin (Ocn), markers for early and terminal OB differentiation, respectively (Dobreva et al., 2006). SATB2 also represses the expression of Hoxa2, a gene that inhibits bone formation (Dobreva et al., 2006). Moreover, SATB2 directly binds to and enhances the transcriptional activity of RUNX2 and ATF4, transcriptional regulators of OB differentiation (Dobreva et al., 2006). Therefore, SATB2 has important roles in controlling OB differentiation.

SATB2 is also involved in neuronal differentiation. Both SATB1 and SATB2 are expressed at specific times in the developing murine central nervous system, although in different cell types (Britanova et al., 2005). Furthermore, SATB2 expression was higher in differentiating neurons and lower in mature neurons (Britanova et al., 2005). SATB2 is also expressed in post-mitotic, differentiating neurons of the rat cerebral cortex (Szemes et al., 2006). Moreover, SATB2 is essential in the development of callosal projection neurons, at least in part by modulating neuronal extension across the corpus callosum to the other cerebral hemisphere by repressing the expression of Ctip2, a gene required for subcortical projection neurons to develop (Alcamo et al., 2008; Britanova et al., 2008). SATB2 binds directly to the MARs located in the Ctip2 locus, recruiting histone deacetylases and altering chromatin configuration.
Moreover, in differentiating cortical neurons, SATB2 has been shown to bind to proteins in the chromatin-remodelling complex (Gyorgy et al., 2008).

In addition, SATB2 has recently been shown to have roles in embryonic stem (ES) cell differentiation. Overexpression of SATB2 in murine ES cells inhibited differentiation and impaired differentiation-associated silencing of Nanog, a regulator of ES cell pluripotency (Savarese et al., 2009). SATB2 expression was associated with increased Nanog expression and the self-renewing fraction of ES cells (Savarese et al., 2009). Moreover, SATB2-deficient ES cells show decreased growth and/or survival compared to wild-type ES cells (Savarese et al., 2009). Taken together, SATB2 not only plays essential roles in palate development, but also in cellular differentiation of OBs, neurons and ES cells. SATB2, therefore, has important developmental functions in a variety of cell types.

1.2.4. Role in cancer

The SATB family was linked to cancer for the first time in a prominent study where SATB1 was linked to breast tumour growth and metastasis (Han et al., 2008). In this study, SATB1 expression was detected in aggressive, metastatic breast cancer cell lines, but not in non-aggressive or normal breast epithelial cells (Han et al., 2008). SATB1 altered the expression of many genes in breast cancer cells, including subsets associated with cell cycle regulation [eg. retinoblastoma gene (RB1) and breast cancer metastasis suppressor 1 (BRMS1)], cell proliferation [eg. transforming growth factor beta 1 (TGFB1) and v-erb-b2 erythroblastic leukemia viral oncogene homolog 2 (ERBB2)], transcriptional regulation (eg. RUNX2), and metastasis [eg. matrix metalloproteinase 2 (MMP2) and MMP9]. SATB1 expression was also associated with poor prognosis (Han et al., 2008). In addition, in a gene expression array, SATB2
was found to be upregulated in canine mammary osteosarcoma (OS; Wensman et al., 2009). Our laboratory has also recently shown that SATB2 mediates chemo-resistance in HNSCC and its expression is highest in advanced-stage HNSCC tumours (Chung et al., 2010). Thus, these early results suggest that the expression of the SATB family proteins play roles in cancer development and progression.

Additional studies, however, have reported conflicting roles for the SATB family in cancer. In contrast to the study by Kohwi-Shigematsu’s group (Han et al., 2008), another group reported that SATB2, instead of SATB1, was associated with increasing tumour grade and poorer overall survival in breast cancer (Patani et al., 2009). In colorectal cancer, downregulation, rather than upregulation, of SATB2 was associated with metastasis and poor prognosis (Wang et al., 2009). Further study with careful discrimination between SATB1 and SATB2 expression is therefore required to delineate the role of the SATB family in cancer and metastasis, and it remains plausible that the expression and activity of the SATB family proteins may have different consequences in different tumours. To this end, our laboratory has undertaken this endeavour by not only studying the role that SATB2 plays in HNSCC, but also in other cancers such as OS.
1.3. **Osteosarcoma**

Terry Fox, a well-known patient and cancer research advocate diagnosed with OS, began the Marathon of Hope with one leg amputated in support of cancer research in 1980. During his run, the cancer metastasized to his lungs and he later succumbed to his disease. Thirty years ago, OS patients underwent amputation and chemotherapy treatment but still had low survival rates (20%). Survival rates have since improved to 65-70% with the use of multi-drug chemotherapy. These rates, however, have been stagnant for the past few years by the lack of new treatment options, especially for patients with metastatic disease, and the causes of OS and factors associated with metastasis are still not well understood. Further research is therefore required to advance our knowledge and understanding of this disease.

1.3.1. **Epidemiology**

OS is the most common type of bone cancer, the 8th most common paediatric cancer, and the 5th most common cancer among adolescents (Ottaviani and Jaffe, 2010; Kim et al., 2010). OS has a higher incidence in males compared to females, and in African American and Hispanic individuals compared to Caucasians (Ottaviani and Jaffe, 2010). OS also has a bimodal age distribution—peaking in the second decade of life, which corresponds to the adolescent growth spur, and in adults older than 65 years of age. OS primary tumours are primarily located near the metaphyseal region of long bones, the area of most active bone growth in childhood—mainly in the femur, tibia and humerus—suggesting a link between OB activity and tumour development (Arndt and Crist, 1999; Kansara and Thomas, 2007). Although OS is the 8th most
common tumour in children, it is the 2\textsuperscript{nd} leading cause of paediatric cancer death (Dass et al., 2006).

### 1.3.2. Diagnosis

At presentation, 85\% of patients experience pain associated with activity and 39\% have a palpable mass during physical examination (Widhe and Widhe, 2000). 20-25\% of OS patients present with metastases, most commonly in the lungs (Bacci et al., 2008). Multifocal bone disease represents the 2\textsuperscript{nd} most common site of OS metastasis. Radiographs and magnetic resonance imaging (MRI) are employed to evaluate both the primary tumour. Computed tomography (CT) is used to detect pulmonary metastases, while a radionuclide bone scan or positron emission tomography (PET) is used to evaluate metastases at other sites. A biopsy obtained by incision or a percutaneous core needle biopsy is used to definitively diagnose OS. Pathologic diagnosis of OS is based on the histological presence of malignant osteoid.

Tumours are staged according to one of two systems: the Musculoskeletal Tumor Society (MSTS) system based on tumour grade, presence of metastases, and tumour extension (Wolf and Enneking, 1996; Enneking et al., 2003), or the American Joint Committee on Cancer system based on tumour size and type of metastases (Greene et al., 2002). The discovery of markers to aid in the identification of OS, as well as prognostic biomarkers, would be very beneficial not only to pathologists, but ultimately to patients as well.

### 1.3.3. Treatment

Prior to the advent of chemotherapy, limb amputation was the primary form of treatment for OS (Dahlin and Coventry, 1967). Currently, OS patients are given multi-drug chemotherapy before
and after they undergo surgery to remove the tumour. These patients are first treated with neoadjuvant (pre-operative) chemotherapy—a combination of doxorubicin, cisplatinum, ifosfamide, etoposide and methotrexate—to shrink the tumour (Bielack et al., 2002; Goorin et al., 2003; Ferrari et al., 2005; Meyers et al., 2005). This initial treatment is followed by surgical resection of the tumour, and in certain circumstances, any metastatic nodules. Limb-sparing surgery and amputation are surgical options, but the specific type of surgery depends on the location, extent of the tumour, as well as patient preference. Following surgery, adjuvant (post-operative) chemotherapy is administered to the patients for approximately six months.

1.3.4. Prognosis

Patient prognosis has improved dramatically since the introduction of chemotherapy for the treatment of OS. Prior to the 1970s, the overall survival rate for non-metastatic OS patients with surgery alone was only 10%. Survival slowly improved as chemotherapy was introduced in the 1980s and current use of multi-agent chemotherapy, together with better surgical techniques, has resulted in increased overall survival rates between 65-70% (Pizzo and Poplack, 2006). However, patients with metastatic OS have a worse prognosis with survival rates of only 10-20% (Wu et al., 2009). It has also been suggested that many non-metastatic OS patients have micrometastases at presentation because in many patients historically treated with surgery alone, recurrence with lung metastases was common, thereby stressing the importance of chemotherapy in long-term patient survival (Dahlin and Coventry, 1967; Rose et al., 2006; Ferrari et al., 2005). Adverse prognostic factors include large tumour size, inadequate surgical margins, poor histological response to neoadjuvant chemotherapy (less than 90% tumour necrosis), axial tumour site (such as spine, ribs, or pelvis), and presence of metastases (Bielack et al., 2002;
Bacci et al., 2006). Recent reports have identified new potential genetic and molecular markers for prognosis, but additional studies are needed to determine whether these independently predict prognosis. For example, increased RUNX2 expression was associated with poor patient survival and metastasis in OS (Won et al., 2009). Deletions of limbic system-associated membrane protein (LSAMP), a putative tumour suppressor in OS, at chromosome 3q13.31 (see below) was also associated with poor survival (Kresse et al., 2009; Yen et al., 2009; Pasic et al., 2010; Sadikovic et al., 2010).

1.3.5. Etiology

The etiologies of OS are still not well understood despite decades of research. Several clinical associations have been identified. As mentioned previously, the development of OS corresponds to the adolescent growth spurt, a period of rapid bone growth. In addition, OS patients were reported to be taller than the general population (Gelberg et al., 1997; Cotterill et al., 2004; Longhi et al., 2005). Age at diagnosis was also earlier in girls than in boys, which corresponds to the earlier age at which girls undergo their growth spurts (Price, 1958; Cotterill et al., 2004). Radiation exposure is the only well-defined risk factor that can result in OS (reviewed in Koshy et al., 2005). However, the latency period from the radiation exposure to the onset of OS is generally long, and since not all patients exposed to radiation develop OS, other factors likely contribute to OS development. Furthermore, only 2% of OS cases can be attributed to radiation exposure (Picci, 2007).

Many cytogenetic abnormalities, including both numerical (gains or losses of chromosomes) and structural (chromosomal rearrangements) alterations, and genomic amplification have been described in OS (reviewed in Sandberg and Bridge, 2003). Many
chromosomes are affected, including those encoding the p53, RB1, MDM2 and RUNX2 genes. Recently, copy number alterations were found in 80% of OS patients at chromosome 3q13.31, which has been identified as the most commonly altered region in this cancer (Pasic et al., 2010).

Specific genetic alterations in the p53 and RB1 tumour suppressor pathways have also been detected in OS. Inactivated or mutated p53 and/or RB1, as well as loss of heterozygosity, have been detected in 70-80% of sporadic tumours (reviewed in Sandberg and Bridge, 2003). Rare hereditary diseases harbouring germline mutations have also been associated with OS predisposition, including patients with germline p53 mutations (Li-Fraumeni syndrome; Malkin et al., 1990; Srivastava et al., 1990; Porter et al., 1992) and RB1 mutations (Hansen et al., 1985; Friend et al., 1986; Ozaki et al., 1993; Chauveinc et al., 2001). Germline p53 mutations were also unexpectedly detected in some patients with sporadic OS (Toguchida et al., 1992; McIntyre et al., 1994; Kleihues et al., 1997). In addition, amplification of MDM2, a negative regulator of p53, has been identified in OS (Miller et al., 1996; Lonardo et al., 1997). MDM2 disrupts the normal function of the p53 pathway by affecting p53 degradation and transcriptional activity. Moreover, the majority of mice in which p53 is specifically deleted in OB progenitor cells developed OS (Lengner et al., 2006). OB-specific deletion of both p53 and RB1 were also sufficient for developing a mouse model that mimics human OS, including the metastatic disease (Walkley et al., 2008; Berman et al., 2008). Interestingly, these studies revealed that unlike mice with p53 deletions or combined p53 and RB1 deletions, mice with homozygous or heterozygous deletions of RB1 did not develop OS, thereby indicating that p53 is important in the initiation of OS development and RB1 acts in synergy with p53 but is not sufficient in current mouse models.

Rothmund-Thomson syndrome (RTS) is a rare autosomal recessive disorder characterized by poikiloderma (skin rash), short stature, skeletal abnormalities, juvenile cataracts
and predisposition to certain cancers, including OS (Larizza et al., 2010). Risk of OS development in RTS patients was associated with the presence of truncating mutations of RECQL4, which encodes a DNA helicase (Wang et al., 2003). RECQL4 is located at chromosome 8q24, a region that frequently undergoes rearrangements and genomic imbalances in OS (Bayani et al., 2003). RECQL4 mRNA is also overexpressed in OS, and this overexpression is associated with structural chromosomal instability (Maire et al., 2009).

More recent studies have linked OS with RUNX2, a regulator of terminal OB differentiation. RUNX2 coordinates progressive growth arrest, culminating in permanent cell cycle exit and terminal OB differentiation, processes which are disrupted in OS (Thomas et al., 2004). In support, changes in differentiation were observed in a large study in which more than 80% of OS cases were described as either poorly differentiated or undifferentiated (Dahlin, 1957). Furthermore, expression of ocn, a marker of terminal OB differentiation, was also low or undetectable in about 75% of OS samples (Hopyan et al., 1999). Several studies have revealed an oncogenic/survival function for RUNX2 in OS. Elevated levels of RUNX2 expression were detected in OS cells (Nathan et al., 2009) and increased RUNX2 expression was associated with poor patient survival, as well as tumour metastasis, in OS (Won et al., 2009). Moreover, RUNX2 was shown to repress the cell cycle gene p21 in OS cells (Westendorf et al., 2002), and also plays a role in the metastatic spread of breast and prostate cancer cells to bone by regulating metastasis-associated genes (reviewed in Pratap et al., 2006). However, a conflicting study reported that RUNX2 induced the pro-apoptotic gene Bax in OS cells (Eliseev et al., 2008) and reveals a pro-death function for RUNX2 in OS. These paradoxical observations seem to indicate an ambiguous role for RUNX2 in OS, but collectively suggest it is deregulated and warrants further investigation.
Therefore, the specific causes of OS remain elusive, however, radiation exposure, specific cytogenetic abnormalities, alterations in the \( p53 \) and \( RB1 \) tumour suppressor pathways, and RUNX2 expression and activity have been implicated. Over the past few decades, little progress has been made in the improvement of overall survival rates, particularly for patients with metastatic disease. Therefore, new therapies and drug targets, as well as further understanding of genes and pathways regulating metastasis, are needed.

1.3.6. Metastasis

20-25% of OS patients present with metastases (Bacci et al., 2008) and the presence of metastases are associated with low overall survival rates (10-20%; Wu et al., 2009; Pizzo and Poplack, 2006). Many non-metastatic OS patients may also have micrometastases at presentation (Dahlin and Coventry, 1967; Rose et al., 2006; Ferrari et al., 2005). Moreover, the presence of metastases is an adverse prognostic factor (Bielack et al., 2002; Bacci et al., 2006; Won et al., 2009). In a frequently-cited review published ten years ago, Hanahan and Weinberg described six hallmarks of cancer, and the sixth hallmark was tissue invasion and metastasis (Hanahan and Weinberg, 2000). In a recent commentary, it was suggested that this sixth hallmark of tissue invasion and metastasis was the sole bona fide hallmark of cancer since only malignant solid tumours are able to invade tissues and metastasize, whereas benign tumours lack this capability (Lazebnik, 2010). Although 90% of human cancer deaths result from metastasis (Sporn, 1996), the mechanisms by which cancer cells escape the primary tumour, invade tissues and form new tumours at distant sites are not completely understood.

Metastasis is a complex process that involves several key steps: invasion and migration, intravasation, circulation, extravasation, and proliferation and angiogenesis (reviewed in Leber
and Efferth, 2009). Each step, along with the major pathways implicated, is briefly described. Cancer cells first detach from the primary tumour and this action is facilitated by the loss of E-cadherin, which maintains cell-cell adhesions. The extracellular matrix (ECM) is degraded by the secretion and activation of proteolytic enzymes, including urokinase plasminogen activator (uPA) and MMPs. uPA regulates MMPs by regulating the activation of several MMP family members. MMPs are able to digest collagen and other components of the ECM. These cancer cells develop lamellipodia, which allow the cells to migrate. Integrins and the Rho family of GTPases, such as Rac, reorganize the actin-cytoskeleton at the front end of these lamellipodia to make new connections with the ECM, while the ones at the distal end are dissolved. The route of cell migration is directed by chemokines and their receptors, such as CXCR4 and CXCL8 (Singh et al., 2007). Interestingly, the phosphatidylinositol-3-kinase (PI3K)/AKT signalling pathway has been implicated in the downregulation of E-cadherin, the activation of proteolytic enzymes, the activation of the Rho family of GTPases, as well as the regulation of chemokines (Jiang and Liu, 2009). Secondly, cancer cells enter blood (or lymphatic) vessels by attaching to the endothelium via adhesion molecules and secreting proteases, such as MMPs, to degrade the endothelium. Thirdly, cancer cells travel to and survive in distant sites. Next, these cells attach to the endothelium of the blood vessels of other organs via adhesion molecules, and degrade the endothelium and basement membrane via proteases. Lastly, the cancer cells proliferate and induce angiogenesis, in part via pathways involving vascular endothelial growth factor (VEGF) and VEGF receptor (VEGFR), to form secondary tumours, thereby completing the metastatic process.

Several genes have been specifically implicated in various steps involved in OS metastasis, including neurofibromatosis-2 (NF2), Ezrin, integrin β4, MMPs, tissue inhibitor of
metalloproteinases (TIMPs) and RUNX2. NF2+/– mice developed various highly metastatic
tumours including OS (McClatchey et al., 1998). The NF2 gene encodes Merlin, a cytoskeletal
protein. Interestingly, Merlin inhibited the p53 degradation mediated by MDM2 via degradation
of MDM2 itself, resulting in increased p53 stability and transcriptional activity (Kim et al.,
2004). High expression of Ezrin, a membrane-cytoskeletal linker protein, was correlated with
poor prognosis in paediatric OS patients (Khanna et al., 2004). Ezrin expression also mediated
the early survival of OS cells that metastasized to the lung (Khanna et al., 2004). In addition,
Ezrin interacts with integrin β4, which functions in cell-matrix adhesion, and integrin β4 has also
been shown to be highly expressed in human tumours and to promote OS metastasis (Wan et al.,
2009). MMPs, proteolytic enzymes that function in protein degradation, were found to be
overexpressed in OS cell lines and this overexpression was associated with metastasis (Bjornland
et al., 2005). Conflicting roles have been reported for both TIMP and RUNX2. TIMP1, a
natural inhibitor of MMPs, has been unexpectedly associated with OS metastasis and cell
survival and growth (Ferrari et al., 2004; Hornebeck et al., 2005). Increased RUNX2 expression
has been associated with poor patient survival and tumour metastasis in OS (Won et al., 2009)
and RUNX2 was reported to repress the cell cycle gene p21 in OS cells (Westendorf et al.,
2002). In contrast, RUNX2 was also reported to induce the pro-apoptotic gene Bax in OS cells
(Eliseev et al., 2008). Therefore, understanding tissue invasion and metastasis is vitally
important in alleviating the burden of metastatic disease in OS patients.
Chapter 2: Hypotheses and rationale

2.1. Role of SATB2 as a novel p73-interacting protein

p73 was identified more than a decade ago (Kaghad et al., 1997) and, as a homologue of the p53 tumour suppressor, has since been intensively studied to determine whether it shares the tumour-suppressor properties of p53. Understanding the genes and signalling pathways involved in regulating p73 is important since different p73 isoforms have specific roles in both development and cancer. During development, ΔNp73 is the predominant p73 isoform expressed in the murine nervous system and, by opposing the pro-apoptotic function of p53 and TAp63, acts as a pro-survival protein in developing neurons (Pozniak et al., 2000; Pozniak et al., 2002; Jacobs et al., 2005). Interestingly, overexpression of ΔNp73 in transgenic mice results in early embryonic lethality, thereby suggesting that the levels of ΔNp73 are important (Erster et al., 2006). Although, in contrast to p53, p73 is rarely mutated in human cancers, TAp73 and ΔNp73 have opposing functions in cancer and the relative levels of these functionally different proteins are highly regulated. ΔNp73 is overexpressed in many tumours, including neuroblastoma, breast, colon and lung cancers (Casciano et al., 2002; Dominguez et al., 2005; Uramoto et al., 2004; Uramoto et al., 2006) and overexpression of ΔNp73 results in transformation of primary cells (Petrenko et al., 2003). TAp73, on the other hand, can activate the transcription of genes involved in cell cycle arrest and apoptosis in response to DNA-damaging agents, including chemotherapeutic drugs, by binding and activating the promoters of numerous p53-target genes (Harms et al., 2004). Moreover, TAp73 is able to induce apoptosis
independent of p53 (Jost et al., 1997; Kaghad et al., 1997; Ishida et al., 2000), which partly accounts for the efficacy of chemotherapy treatment against tumours with mutated or inactivated p53 (Irwin et al., 2003). A role for TAp73 as a tumour suppressor protein is supported by the high incidence of tumour development in TAp73-deficient mice (Tomasini et al., 2008). In support, decreased levels of TAp73 are detected in leukemias/lymphomas and bladder cancer (Corn et al., 1999; Kawano et al., 1999; Puig et al., 2003; Park et al., 2000).

Because all members of the p53 family share many structural similarities, they all have overlapping, as well as distinct, functions. Nevertheless, it is still not well understood how p53, p63 and p73 are differentially regulated. To further add to the complexity, all three family members have variants derived from both alternative splicing and differential promoter utilization. In particular, p73 has many isoforms generated at both the N- and C-terminus, resulting in tumour-suppressor-like (pro-apoptotic) TAp73 and oncogenic (anti-apoptotic) ΔNp73 isoforms, as well as p73α-η variants (Murray-Zmijewski et al., 2006). The mechanisms by which the various p73 isoforms are specifically regulated leading to the observed expression patterns in specific cells (eg. tumour cells) are also not well understood. In addition, ΔNp73 inhibits and is also induced by p53 and TAp73, thereby forming a dominant-negative feedback loop (Grob et al., 2001; Kartasheva et al., 2002; Nakagawa et al., 2002; Vossio et al., 2002). ΔNp63 has also been shown to inhibit TAp73 in HNSCC by heterocomplex formation (Rocco et al., 2006; Chung et al., 2010). This interrelationship among p53, p63 and p73 further illustrates the complexity of regulating specific p53 family isoforms. However, several proteins have been identified that differentially regulate p53, p63 and p73. MDM2 mediates the ubiquitination and subsequent proteasomal degradation of p53 (Haupt et al., 1997; Honda et al., 1997; Kubbutat et al., 1997), but its interaction with p73 results in the stabilization and inhibition of the
transcriptional activity of p73 (Balint et al., 1999; Dobbelstein et al., 1999; Zeng et al., 1999). ITCH promotes the ubiquitination and subsequent proteasomal degradation of p73 and p63, but does not interact with p53 (Rossi et al., 2005; Rossi et al., 2006). Moreover, ITCH only acts on the α and β, but not the γ or δ, isoforms of p73 (Rossi et al., 2005). In addition, PIAS1 only sumoylates the α variant of p73, resulting in its proteasomal degradation and inhibition of its transcriptional activity (Minty et al., 2000; Munarriz et al., 2004).

Therefore, the initial broad questions that we wanted to address include: (1) what makes p73 different from the other p53 family members, p53 and p63? (2) what accounts for the specific functions of p73? and (3) what proteins/pathways differentially regulate p73 N- and C-terminal isoforms? We hypothesized that the activity of p73, like many transcription factors, may be modulated by binding to unique regulatory proteins. To begin this search, the C-terminus of p73, which lacks the TAD and DBD and the region most divergent from that of p53, was used to find proteins that bind specifically to p73. SATB2 was identified by our laboratory as a novel p73-interacting protein. SATB2 regulates gene expression either by binding directly to nuclear MARs of target genes, or by binding and enhancing the transcriptional activity of other transcription factors. MARs are regulatory DNA sequences that are involved in higher-order chromatin organization, which is important in the regulation of gene expression, and in turn, cellular differentiation. SATB2 plays important roles in craniofacial patterning and OB differentiation (FitzPatrick et al., 2003; Britanova et al., 2006; Dobreva et al., 2006), as well as neuronal differentiation (Britanova et al., 2005; Szemes et al., 2006; Alcamo et al., 2008; Britanova et al., 2008; Gyorgy et al., 2008). The SATB family has also been linked to several cancers, such as breast cancer (Han et al., 2008; Patani et al., 2009), OS (Wensman et al., 2009), colorectal cancer (Wang et al., 2009), and HNSCC (Chung et al., 2010). In particular, our
laboratory has recently shown that SATB2 mediates chemo resistance in HNSCC and its 
expression is highest in advanced-stage HNSCC tumours (Chung et al., 2010). SATB2, 
therefore, has important functions in cancer and development.

It is still not well understood how the p53 family members, and p73 isoforms 
themselves—having similar structure and overlapping roles—are differentially regulated. We 
hypothesized that SATB2 may be a regulatory protein that binds specifically to certain p73 
isoforms, thereby modulating p73 activity. By studying the interaction between p73 and SATB2, 
we expected to enhance our understanding of the function and regulation of p73. These studies 
would help us to understand the regulatory mechanisms for p73 activities, including the 
induction of apoptosis in the presence of various chemotherapy agents. Moreover, since both 
proteins are independently involved in both cancer and neuronal development, their interaction 
may play a role in these processes. In this study, I report that SATB2 interacts with certain p73 
C-terminal isoforms, and affects the transcriptional activity and stability of these isoforms 
independent of binding. Ultimately, these studies may help to elucidate the unique regulatory 
pathways for p73 and SATB2 in cancer and development.
2.2. Role of SATB2 in osteosarcoma and invasion

Overall survival rates for OS patients have not changed significantly over the past few years with few new treatment options, especially for those with metastatic disease. Furthermore, the genetic factors involved in OS pathogenesis are still not well understood. Improved chemotherapies and surgical techniques have increased the overall survival rates to 65-70% (Pizzo and Poplack, 2006). Nevertheless, 20-25% of OS patients present with metastases, most commonly in the lungs (Bacci et al., 2008), and these patients are rarely curable. OS primarily occurs near the area of most active bone growth in childhood, suggesting a link between OB activity and tumour development (Arndt and Crist, 1999; Kansara and Thomas, 2007). The specific causes of OS remain elusive; however, radiation exposure, specific cytogenetic abnormalities, alterations in the $p53$ and $RB1$ tumour suppressor pathways, and RUNX2 (which has been linked with SATB2) expression and activity have been implicated (reviewed in Ta et al., 2009). Therefore, new therapies and drug targets, especially for metastatic disease, are needed.

I detected high levels of SATB2 in Saos-2, an OS cell line, and hypothesized that SATB2 may be involved in cancer and metastasis for the following reasons. Studies (albeit conflicting) have already implicated the SATB family in tumourigenesis and metastasis. Breast tumour growth and metastasis were promoted by SATB1, which was more highly expressed in aggressive, metastatic breast cancer cell lines and also altered the gene expression profile in breast cancer cells (Han et al., 2008). Our laboratory has also recently shown that SATB2 mediates chemoresistance in HNSCC and its expression is highest in advanced-stage HNSCC tumours (Chung et al., 2010). Thus, I specifically wanted to investigate a role for SATB2 in OS for several reasons. Firstly, $SATB2$ has already been shown to be upregulated in canine OS in a
gene expression array (Wensman et al., 2009). Secondly, in comparison to numerous cell lines, we detected the highest levels of endogenous SATB2 in OS cell lines. Higher levels of SATB2 may indicate that this protein is overexpressed or upregulated in OS versus other cell types.

Lastly, the OB is the cell of origin for OS and SATB2 controls OB differentiation by regulating OB-specific genes, such as Bsp, Ocn and Hoxa2, and by enhancing the transcriptional activity of OB differentiation regulators, such as RUNX2 and ATF4 (Dobreva et al., 2006). These observations, together with the published role of SATB1 in breast cancer, suggest that SATB2 may play a role in OS and metastasis.

As noted above, metastasis is a critical indicator of more aggressive OS tumours. Tissue invasion and metastasis are considered the most important hallmark since only malignant solid tumours are able to invade tissues and metastasize, but benign tumours lack this capability (Lazebnik, 2010). Although, 90% of human cancer deaths result from metastasis (Sporn, 1996), the mechanisms by which cancer cells escape the primary tumour, invade tissues and form new tumours at distant sites are not well understood. Since several studies have linked the expression of the SATB family proteins to cancer development and progression, and specifically to the regulation of genes implicated in metastasis (eg. MMPs), our aim was to study whether SATB2 may be involved in OS, and possibly metastasis. We planned to examine the levels of SATB2 expression in human OS cell lines and tumour samples, and utilize different types of invasion and migration assays to assess whether SATB2 plays a role in OS and metastasis. I hypothesized that SATB2 expression may be elevated in both OS cell lines and OS tumour samples since high levels have already been observed in the Saos-2 OS cell line and in canine OS (Wensman et al., 2009). We also hypothesized that SATB2 may be involved in OS metastasis since SATB2 has already been implicated in the metastasis of colorectal cancer cells (Wang et al., 2009) and its
family member, SATB1, has been shown to promote breast cancer metastasis (Han et al., 2008). By undertaking this study, we endeavour to further our knowledge and understanding of OS and pathways involved in metastasis in the hopes that novel therapies or drug targets may be developed that will improve the long-term survival and quality of life for many children, especially those afflicted by metastatic disease.
Chapter 3:
Role of SATB2 as a novel p73-interacting protein

The work presented in this chapter was completed by me, except for Figure 3.1A by Dr. Meredith Irwin and Lynn Cheng, and Figures 3.3 to 3.5 by Lynn Cheng.

3.1. Introduction

p73 was identified more than a decade ago (Kaghad et al., 1997) as a homologue of the p53 tumour suppressor protein and, has since been intensively studied for tumour-suppressor-like properties. The structure of p73 consists of four functional domains: the TAD, DBD, OD and SAM domain (Chapter 1, Figure 1.1). p73 isoforms are derived from both alternative splicing and differential promoter utilization. In general, p73 variants that contain the TAD are denoted by TAp73, while those that are derived from the internal cryptic promoter and lack this N-terminal TAD are denoted by ΔNp73. TAp73 can activate the transcription of genes involved in cell cycle arrest and apoptosis in response to DNA-damaging agents, including chemotherapeutic drugs, by binding and activating the promoters of numerous p53-target genes (Harms et al., 2004). ΔNp73 inhibits and is also induced by p53 and TAp73, thereby forming a dominant-negative feedback loop (Grob et al., 2001; Kartasheva et al., 2002; Nakagawa et al., 2002; Vossio et al., 2002). Alternative splicing at the C-terminus of p73 gives rise to several C-terminal splice variants. The two most commonly detected p73 splice variants are p73α and β. p73α, but not p73β, contains a SAM domain and although both TAp73α and β share many
activities and functions of p53, TAp73β is more potent at activating the transcription of many target genes and inducing apoptosis (Jost et al., 1997; Zhu et al., 1998; Di Como et al., 1999; Lee and La Thangue, 1999).

p73 plays essential roles in both development and cancer. ΔNp73 is the predominant isoform of p73 that is expressed in the developing murine nervous system and, by opposing the apoptotic function of p53 and TAp63, acts as a pro-survival protein in cortical neurons (Pozniak et al., 2000; Pozniak et al., 2002; Jacobs et al., 2005). The overexpression of ΔNp73 in transgenic mice results in early embryonic lethality, suggesting the levels of ΔNp73 are important (Erster et al., 2006). Although p73 is rarely mutated in human cancers unlike p53, TAp73 and ΔNp73 have opposing functions in cancer. ΔNp73 is overexpressed in many tumours, including neuroblastoma, breast, colon and lung cancers (Casciano et al., 2002; Dominguez et al., 2005; Uramoto et al., 2004; Uramoto et al., 2006). In contrast, in response to chemotherapeutic and DNA-damaging agents, TAp73 induced and mediates apoptosis independent of p53 (Jost et al., 1997; Kaghad et al., 1997; Ishida et al., 2000), which partly accounts for the efficacy of chemotherapy treatment against tumours with mutated or inactivated p53 (Irwin et al., 2003). A role for TAp73 as a tumour suppressor is supported by the high incidence of spontaneous tumour development in TAp73-deficient mice (Tomasini et al., 2008).

In order to identify proteins that bind to and regulate p73, our laboratory performed large-scale immunoprecipitations followed by mass spectrometry, and identified SATB2 as a novel p73α-interacting protein. SATB2 has two CUT domains and one homeodomain (Chapter 1, Figure 1.4). In addition, the N-terminus of the SATB2 homologue, SATB1, contains a dimerization domain that is highly homologous to PDZ domains (Galande et al., 2001), which may play a role in protein-protein interactions. SATB2 also undergoes sumoylation by PIAS1,
which has been shown to potentially regulate subcellular localization (Dobreva et al., 2003). SATB2 regulates gene expression either by binding directly to nuclear MARs of target genes, or by binding and altering the transcriptional activity of other transcription factors, acting as a co-activator or co-repressor. MARs are regulatory DNA sequences that are involved in higher-order chromatin organization, which is important in the regulation of gene expression, and in turn, cellular differentiation.

SATB2 plays essential roles in craniofacial patterning and OB differentiation (FitzPatrick et al., 2003; Britanova et al., 2006; Dobreva et al., 2006), and is also involved in neuronal differentiation (Britanova et al., 2005; Szemes et al., 2006; Alcamo et al., 2008; Britanova et al., 2008; Gyorgy et al., 2008). The SATB family has also been linked to several cancers, such as breast cancer (Han et al., 2008; Patani et al., 2009), OS (Wensman et al., 2009), colorectal cancer (Wang et al., 2009), and HNSCC (Chung et al., 2010). In particular, our laboratory has recently shown that SATB2 mediates chemoresistance in HNSCC and its expression is highest in advanced-stage HNSCC tumours (Chung et al., 2010). Therefore, SATB2 has roles in both cancer and development.

It is still not well understood how the p53 family members, and specifically different N- and C-terminal p73 isoforms—having similar structure and overlapping roles—are differentially regulated. We hypothesized that differential binding to regulatory proteins might account for some of those differences. To date, several interacting proteins have been identified. MDM2 mediates the ubiquitination and subsequent proteasomal degradation of p53 (Haupt et al., 1997; Honda et al., 1997; Kubbhat et al., 1997), and although it can promote the ubiquitination and neddylation of p73, this results in the stabilization and inhibition of the transcriptional activity of TAp73 (Balint et al., 1999; Dobbelstein et al., 1999; Zeng et al., 1999; Watson et al., 2006).
ITCH promotes the ubiquitination and subsequent proteasomal degradation of p73, but does not interact with p53 (Rossi et al., 2005). Moreover, ITCH only binds to p73α and β, but not γ or δ, isoforms (Rossi et al., 2005). PIAS1 only sumoylates the α variant of p73, resulting in its proteasomal degradation and inhibition of its transcriptional activity (Minty et al., 2000; Munarriz et al., 2004). In this study, I report that SATB2 interacts only with certain p73 C-terminal isoforms, affecting the transcriptional activity and stability of these isoforms.
3.2. Materials and methods

3.2.1. Cell Culture
Saos-2 OS, HeLa cervical cancer and 293 human embryonic kidney (HEK) cell lines were obtained from American Type Culture Collection (ATCC; Rockville, MD) and the SCC9 HNSCC cell line was generously provided by Dr. Suzanne Kamel-Reid. Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco; Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Hyclone; Logan, UT) and maintained at 37°C in a humidified 5% CO₂ incubator.

3.2.2. Plasmids and transfection
pcDNA3-T7-p73-DDα, pcDNA3-T7-p73-DDβ and pcDNA3-hemagglutinin (HA)-TAp73α (L371P) were described previously (Irwin et al., 2000). pcDNA3-HA-TAp73α, pcDNA3-HA-TAp73β, pcDNA3-HA-TAp73γ, pcDNA3-HA-TAp73δ, and pcDNA3-HA-ΔNp73α were generously provided by Dr. Gerry Melino. pcDNA3-T7-SATB2 was described previously (Chung et al., 2010). pcDNA3-T7-SATB2-mut, a T7-tagged SATB2 double mutant at sumoylation sites K233R and K350R, was generated by PCR with primers 5’-GGT ATA AAA AGT ACA AGA AGA TTA GAG TGG AAA GAG TGG AAC GAG-3’ and 5’-CTC GTT CCA CTC TTT CCA CTC TAA TCT TCT TGT ACT TTT TAT ACC-3’ for K233R and 5’-CCC AGA GCC GTC TGG CCA GCC ACC-3’ and 5’-GGT TGG CTC TGG CCT AAC TGC TCT GGG-3’ for K350R using the QuikChange Site-Directed Mutagenesis Kit (Stratagene; La Jolla, CA). pcDNA3-T7-SATB2ΔC (aa 1-300), a T7-tagged SATB2 mutant lacking the C-
terminus, was generated by PCR with primers 5’-GAC GAC GGA TCC GAG CGG CGG AGC GAG AGC CCG-3’ and 5’-GAC GAC GAA TTC TTA GGG AGA AAG AAG ACC AGG-3’, digestion with BamHI and EcoRI, and ligation into pcDNA3 vector. pcDNA3-T7-SATB2ΔN (aa 301-626), a T7-tagged SATB2 mutant lacking the N-terminus, was similarly generated using primers 5’-GAC GAC GGA TCC CAG CTT AGT CCA CAA CTT GTA-3’ and 5’-GAC GAC GAA TTC TTA CAG GGC TTC TAA GGA GAT-3’. pcDNA3-T7-SATB2ΔPDZ, a T7-tagged SATB2 mutant lacking the PDZ-like domain, was generated by PCR with primers 5’-GAC GAC GAA TTC ATG GCT AGC ATG ACT GGT G-3’ and 5’-CGC AGG CAA GTC TTC CAA GTC ATA TTC AAG AGA GCC-3’ amplifying the region before the PDZ-like domain and 5’-GGC TCT CTT GAA TAT GAC TTG GAA GAC TTG CCT GCG-3’ and 5’-GAC GAC GAC CTC GAA GTT ATC TCT GGT CAA TTT C-3’ amplifying the region after the PDZ-like domain, digestion with EcoRI and XhoI, and ligation into pcDNA3 vector. Cells were transfected using either the FuGENE 6 (Roche; Indianapolis, IN) or polyethylenimine (PEI; Polysciences; Warrington, PA) method.

3.2.3. Antibodies

Polyclonal anti-p73 (2301) antibody was described previously (Watson et al., 2006). Polyclonal anti-TAp73 (BL906 and H-79) antibodies were obtained from Bethyl Laboratories (Montgomery, TX) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Monoclonal anti-p73 (ER-15) antibody was described previously (Marin et al., 1998). Monoclonal anti-p73 (GC-15) antibody was obtained from Oncogene Research (La Jolla, CA). Polyclonal anti-SATB2 (ab34735) antibody was obtained from Abcam (Cambridge, UK). Polyclonal anti-SATB2-CT antibody was generated by our laboratory and described previously (Chung et al.,
Polyclonal anti-HA antibody was obtained from Novus Biologicals (Littleton, CO). Monoclonal anti-HA (12CA5) and monoclonal anti-T-antigen (Pab419) hybridoma supernatants were prepared as described previously (Harlow et al., 1981; Harlow and Lane, 1988). Monoclonal anti-T7 antibody was obtained from Novagen (San Diego, CA). Polyclonal anti-pan-actin was obtained from Cell Signaling (Danvers, MA). Monoclonal anti-α-tubulin (B-5-1-2) antibody was obtained from Sigma-Aldrich (St Louis, MO). Monoclonal anti-vinculin (V284) antibody was obtained from Upstate (Billerica, MA).

### 3.2.4. Immunoprecipitations and Immunoblotting

Cells in 10-cm tissue culture plates were scraped, centrifuged, and washed in cold phosphate-buffered saline (PBS). These cells were then lysed in EBC lysis buffer (50 mM Tris [pH 8.0], 120 mM NaCl, 0.5% Nonidet P-40) supplemented with complete protease inhibitors (Roche; Indianapolis, IN) at 4°C for 30 min. Samples were centrifuged at 4°C for 10 min to remove cell debris. The Bradford method (Bio-Rad; Hercules, CA) was used to determine protein concentrations. 10% of the whole cell extracts was used for inputs. Immunoprecipitations (IPs) were performed with the indicated antibody and Protein A Sepharose (GE Healthcare; Piscataway, NJ) at 4°C for 2h and then washed five times with NETN (20 mM Tris [pH 8.0], 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40) buffer. Samples were boiled in sample buffer, proteins were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane (Bio-Rad; Hercules, CA). Membranes were blocked in Tris-buffered saline (TBS) supplemented with 5% (wt/vol) powdered milk for 1 h at room temperature and incubated with primary antibody at 4°C overnight. Membranes were then washed five times with TBS with Tween 20 (TBST) for 5 min at room temperature and
incubated with horseradish peroxidase-conjugated goat anti-mouse or -rabbit secondary antibody (Pierce Biotechnology; Rockford, IL) in TBS supplemented with 2% (wt/vol) powdered milk for 1 h at room temperature. Membranes were again washed five times with TBST and bound secondary antibody was detected by enhanced chemiluminescence with Supersignal (Pierce Biotechnology; Rockford, IL). Unless otherwise stated, all IP/IB experiments were performed at least three independent times.

3.2.5. Semi-quantitative RT-PCR

Total RNA was extracted with TRIzol Reagent (Invitrogen; Carlsbad, CA) and converted into cDNA with GeneAmp: Gold RNA PCR Core Kit (Applied Biosystems; Foster City, CA). Reverse transcription-polymerase chain reaction (RT-PCR) was performed with Taq DNA polymerase (Qiagen; Mississauga, ON) using the following conditions: initial denaturation at 94°C for 3 min, 34-35 cycles of denaturation at 94°C for 45 s, annealing at 50°C for 45 s and extension at 72°C for 1 min, and a final extension at 72°C for 10 min. Primer sequences are as follows: *PUMA* 5’-TCA ACG CAG CAG TAC GAG CGG-3’ and 5’-GTA AGG GCA GGA GTC CCA TG-3’, *p21* 5’-GGA AGA CCA TGT GGA CCT GT-3’ and 5’-TTA GGG CTT CCT TTT GGA GA-3’, and *β-actin* 5’-CTG GAA CGG TGA AGG TGA CA-3’ and 5’-AAG GGA CTT CCT GTA ACA ATG-3’. mRNA levels were quantified by densitometry using ImageJ (http://rsbweb.nih.gov/ij/). *PUMA* and *p21* mRNA levels were then normalized to *β-actin* (internal control) levels and expressed as fold change.
3.2.6. Protein half-life

Approximately 48 h post-transfection, cells were treated with 20 μg/mL cycloheximide (CHX; Sigma-Aldrich; Oakville, ON) and harvested at various time points. TAp73 protein levels were quantified by densitometry using ImageJ, normalized to α-tubulin (loading control) levels and expressed as percent change.

3.2.7. Adenoviral infection

Viral work was performed in accordance with the safety guidelines of The Hospital for Sick Children (Toronto, ON). Adenovirus expressing SATB2 and control GFP (Ad-SATB2 and Ad-GFP, respectively) were obtained from Vector Biolabs (Philadelphia, PA) as previously described (Chung et al., 2010). The titre of the adenovirus was determined by Vector Biolabs using several titration procedures, including the plaque formation assay. In this assay, a monolayer of 293 HEK cells are infected with serial dilutions of the virus, leading to the formation of plaques, and the viral titre is then determined to be the number of plaques per ml of media. SCC9 cells were infected at 50 multiplicity of infection (MOI) the day after seeding. Cells were then lysed and immunoblotted as described above.

3.2.8. Proteasomal inhibition

Approximately 48 h post-transfection, cells were treated with 20 μM of the proteasome inhibitor MG-132 (Boston Biochem; Cambridge, MA) for 6 h. Cells were then lysed and immunoblotted as described above.
3.3. Results

3.3.1. SATB2 binds to p73

We hypothesized that the activity of p73, like many transcription factors, may be modulated by binding to unique regulatory proteins. To identify p73-binding partners, our laboratory previously utilized N-terminally truncated forms of p73 (p73-DD\(\alpha\) and p73-DD\(\beta\); Irwin et al., 2000) lacking the TAD and the DBD such that only p73 C-terminal-specific binding partners would be identified. The C-terminus was chosen because this is the region most divergent from that of p53. These truncation mutants were overexpressed in Saos-2 (p53\(^{-/-}\)) cells and lysates were immunoprecipitated with anti-p73, anti-T7 and anti-T-antigen (control) antibodies. Silver staining revealed the presence of several unique bands including a 100-kDa protein that was immunoprecipitated by the anti-p73, but not by the control, antibody (Figure 3.1A). This band was isolated, sent for analysis by mass spectrometry, and identified as KIAA1034. In 2003, using different screening methods, two groups independently reported KIAA1034 to be SATB2 (Dobreva et al., 2003; FitzPatrick et al., 2003).

In order to validate the interaction identified by mass spectrometry, full-length p73 isoforms (rather than truncation mutants) were utilized. Single as well as co-transfections of plasmids encoding either HA-TAp73\(\alpha\) or \(\beta\) and T7-SATB2 were first carried out in Saos-2 cells. Cells were lysed, followed by IP with an anti-HA (12CA5) antibody. Bound proteins were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and the immunoblot (IB) was probed with an anti-HA and anti-T7 antibody (Figure 3.1B). SATB2 was detected only in cells co-transfected with plasmids encoding T7-SATB2 and HA-TAp73\(\alpha\) (lane 11), but not HA-
TAp73β (lane 12), indicating that SATB2 interacts with exogenous TAp73α, but not with TAp73β.

To ask whether SATB2 also binds to ΔNp73 isoforms, plasmids encoding T7-SATB2 and HA-TAp73α (positive control) or HA-ΔNp73α were utilized in similarly-performed co-IP experiments (Figure 3.1C). SATB2 binding was detected when it was co-expressed with ΔNp73α (lane 12). To examine the interaction of SATB2 with other C-terminal p73 isoforms, similar co-IP experiments were carried out using plasmids encoding HA-TAp73α (positive control), β (negative control), γ or δ and T7-SATB2 (Figure 3.1D). Interestingly, SATB2 additionally co-immunoprecipitated with TAp73γ (lane 19), but not with TAp73δ (lane 20). Thus, SATB2 binds to TAp73α and γ, but not to TAp73β or δ. Taken together, these data suggest that SATB2 interacts with certain C-terminal isoforms of p73 irrespective of the N-terminus.

SATB2 has been shown to undergo sumoylation at two lysine sites (K233 and K350), and since mutation of these lysines into arginines has been reported to result in enhanced SATB2 binding to MARs, increased activity, and altered localization in the nucleus (Dobreva et al., 2003), I asked whether sumoylation of SATB2 affects its ability to bind to p73. Thus, a plasmid encoding a T7-tagged SATB2 double mutant at sumoylation sites K233R and K350R (T7-SATB2-mut) was generated. Saos-2 cells were transiently transfected with plasmids encoding HA-TAp73α and either T7-SATB2 (positive control) or T7-SATB2-mut. These cells were lysed and immunoprecipitated with an anti-HA antibody. Bound proteins were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted with anti-T7 and anti-HA antibodies (Figure 3.2). SATB2-mut was detected in cells co-transfected with plasmids encoding
T7-SATB2-mut and HA-TAp73α (lane 12), thereby suggesting that SATB2 binding to TAp73α is not affected by its inability to be sumoylated.

Since our initial objective was to search for specific p73 regulatory proteins, we assessed whether SATB2 could also bind to p53 in addition to p73. Co-IP experiments were similarly performed using plasmids encoding HA-TAp73α (positive control), HA-TAp73β (negative control), HA-p53 and T7-SATB2 (Figure 3.3). Binding was observed when SATB2 was co-expressed with TAp73α, but not with TAp73β or p53 (lane 16), thereby revealing that SATB2 does not interact with p53. When SATB2 is co-expressed with TAp73β or p53, decreased SATB2 levels are frequently observed in the inputs (eg. lanes 7 and 8, respectively). In order to determine whether the absence of binding to p53 or TAp73β is due to low levels of SATB2, we have attempted to equalize SATB2 levels by altering the amounts of plasmids transfected, but have still been unable to demonstrate an interaction between SATB2 with either TAp73β or p53 (data not shown). We also investigated whether the other SATB family member, SATB1, could bind to p73. In co-IP experiments using plasmids encoding HA-TAp73α, T7-SATB1 and T7-SATB2 (Figure 3.4), we observed that TAp73α did not co-immunoprecipitate with SATB1 (lane 5). Therefore, SATB2 does not bind to p53, nor does p73 bind to SATB1.

To determine whether SATB2 binds to p73 under physiological conditions, an endogenous approach was used. 293 HEK cells were lysed and immunoprecipitated with various anti-p73 and anti-pan-actin (negative control) antibodies. Bound proteins were resolved by SDS-PAGE, and immunoblotted with anti-SATB2 and anti-p73 antibodies (Figure 3.5). SATB2 was immunoprecipitated by all anti-p73 antibodies used (lanes 3-5), but not by the control antibody (lane 2), thereby validating that endogenous SATB2 binds to endogenous TAp73. Note that the SATB2 protein pulled down using the anti-p73 antibodies (lanes 3-5) co-migrates with a higher-
migrating band in the input (lane 1) and may indicate that the immunoprecipitated SATB2 protein may be modified forms. Thus, SATB2 and p73 form a complex in cells when overexpressed as well as at the endogenous level.
Figure 3.1

A. IP:

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<tr>
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B. Input  | HA IP
---|---
HA-TAp73α | - + - - + - - - - + -
HA-TAp73β | - - + - - + - - + - - +
T7-SATB2  | - - + + + - - + + +

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T7 IB

HA IB
Figure 3.1

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<td>T7-SATB2</td>
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Figure 3.1. SATB2 binds to exogenous p73. (A) Whole cell extracts from stable clones of Saos-2 cells with plasmids encoding vector alone, T7-p73-DD\(\alpha\) (C-terminus of p73\(\alpha\)) or T7-p73-DD\(\beta\) (C-terminus of p73\(\beta\)) were immunoprecipitated with anti-p73 (ER-15), anti-T7 or anti-T-antigen (control) antibodies. Immunoprecipitations were resolved on SDS-PAGE and stained with silver nitrate. The identity of the band at approximately 100 kDa was determined by mass spectrometry to be KIAA1034 (later identified as SATB2). (B) Saos-2 cells were transiently transfected with plasmids encoding HA-TA\(\alpha\) or \(\beta\) and T7-SATB2. 10% of the whole cell extracts was used for inputs, while the remainder was immunoprecipitated with an anti-HA (12CA5) antibody and immunoblotted with anti-T7 and anti-HA antibodies. (C) Saos-2 cells were transiently transfected with plasmids encoding HA-TA\(\alpha\), HA-\(\Delta\)Np73\(\alpha\) and T7-SATB2. 10% of the whole cell extracts was used for inputs, while the remainder was immunoprecipitated with an anti-HA (12CA5) antibody and immunoblotted with anti-T7 and anti-HA antibodies. (D) Saos-2 cells were transiently transfected with plasmids encoding HA-TA\(\alpha\), \(\beta\), \(\gamma\) or \(\delta\) and T7-SATB2. 10% of the whole cell extracts was used for inputs, while the remainder was immunoprecipitated with an anti-HA (12CA5) antibody and immunoblotted with anti-T7 and anti-HA antibodies.
Figure 3.2. p73 binds to SATB2-mut. Saos-2 cells were transiently transfected with plasmids encoding HA-TAp73α and T7-SATB2 or T7-SATB2-mut (mutated at sumoylation sites K233R and K350R). 10% of the whole cell extracts was used for inputs, while the remainder was immunoprecipitated with an anti-HA (12CA5) antibody and immunoblotted with anti-T7 and anti-HA antibodies.
Figure 3.3. SATB2 does not bind to p53. OS cells were transiently transfected with plasmids encoding HA-TAp73α, β, HA-p53 and T7-SATB2. 10% of the whole cell extracts was used for inputs, while the remainder was immunoprecipitated with an anti-HA (12CA5) antibody and immunoblotted with anti-T7 and anti-HA antibodies.
Figure 3.4. p73 does not bind to SATB1. Saos-2 cells were transiently transfected with plasmids encoding HA-TAp73α, T7-SATB1 and T7-SATB2. 10% of the whole cell extracts was used for inputs, while the remainder was immunoprecipitated with an anti-HA (12CA5) antibody and immunoblotted with anti-T7 and anti-HA antibodies.
Figure 3.5. SATB2 binds to endogenous p73. Whole cell extracts from 293 HEK cells were immunoprecipitated with various anti-p73 and anti-pan-actin (negative control) antibodies, and immunoblotted with anti-SATB2-CT and anti-p73 antibodies. 75 mg of the whole cell extracts were used as an input.
3.3.2. Mapping of SATB2 and p73 regions required for binding

In order to determine the region of SATB2 that is required for binding to p73, a plasmid encoding a T7-tagged SATB2 truncation mutant lacking the C-terminus (T7-SATB2ΔC) and another lacking the N-terminus (T7-SATB2ΔN) were generated. HeLa cells were transiently transfected with plasmids encoding HA-TAp73α and either T7-SATB2 (positive control), T7-SATB2ΔC or T7-SATB2ΔN. These cells were lysed and immunoprecipitated with an anti-HA (12CA5) antibody. Bound proteins were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted with anti-T7 and anti-HA antibodies (Figure 3.6A). Binding was only observed when TAp73α was co-expressed with SATB2ΔC (lane 5), thereby supporting a role for the N-terminus of SATB2 containing the region to which TAp73α binds.

The N-terminus of SATB2 contains a PDZ-like domain, and since PDZ domains are often involved in protein-protein interactions, I examined whether p73 binds to the PDZ-like domain of SATB2. Thus, a plasmid encoding a T7-tagged SATB2 mutant lacking the PDZ-like domain (T7-SATB2ΔPDZ) was generated. Saos-2 cells were transiently transfected with plasmids encoding HA-TAp73α and either T7-SATB2 (positive control) or T7-SATB2ΔPDZ. These cells were lysed and immunoprecipitated with an anti-HA antibody. Bound proteins were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted with anti-T7 and anti-HA antibodies (Figure 3.6B). SATB2ΔPDZ was not detected in cells co-transfected with plasmids encoding T7-SATB2ΔPDZ and HA-TAp73α (lane 12), thereby suggesting that the PDZ-like region of SATB2 is necessary for binding to p73.

Since the initial search for p73-binding proteins was performed using plasmids encoding N-terminally truncated forms of p73 lacking the TAD and the DBD (p73-DDα and p73-DDβ), I hypothesized that amino acids in the C-terminus of p73 may be required for binding to SATB2.
In order to narrow down the region of the C-terminus that is required, HeLa cells were transiently transfected with plasmids encoding T7-SATB2 and HA-TAp73α (positive control), HA-ΔNp73α (lacking the TAD), T7-p73-DDα (lacking the TAD and DBD), HA-TAp73γ (lacking the SAM domain) or HA-TAp73α (L371P). HA-TAp73α (L371P) is a point mutant in the OD that abrogates p73 oligomerization (Irwin et al., 2000). These cells were lysed and immunoprecipitated with an anti-HA (12CA5) antibody. Lysates of cells that had been transfected with T7-p73-DDα were immunoprecipitated with an anti-p73 (ER-15) antibody. Bound proteins were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted with anti-T7 and anti-HA antibodies (Figure 3.6C). SATB2 is able to bind to all of the tested forms of p73 (lanes 6-10; binding in lane 9 was observed at darker exposures), thereby suggesting that the TAD, DBD, SAM domain, and a functional OD of p73 are not required for binding to SATB2. Hence, the C-terminus of p73 and the PDZ-like domain of SATB2 appear to be essential for binding.
Figure 3.6

A. 

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<tbody>
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<td>T7-SATB2ΔN</td>
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| 95 | 72 | 34 |

T7 IB (SATB2)

HA IB

T7 IB (SATB2ΔC/N)

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T7 IB

HA IB

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T7 IB (SATB2)

HA IB

T7 IB (p73-DDα)
Figure 3.6. SATB2 and p73 binding regions.  (A) HeLa cells were transiently transfectected with plasmids encoding HA-TAp73α, T7-SATB2, T7-SATB2ΔC and T7-SATB2ΔN.  10% of the whole cell extracts was used for inputs, while the remainder was immunoprecipitated with an anti-HA (12CA5) antibody.  Two IPs were loaded per lane and proteins were immunoblotted with anti-T7 and anti-HA antibodies.  (B) Saos-2 cells were transiently transfected with plasmids encoding HA-TAp73α and T7-SATB2 or T7-SATB2ΔPDZ (lacking the PDZ-like domain).  10% of the whole cell extracts was used for inputs, while the remainder was immunoprecipitated with an anti-HA (12CA5) antibody and immunoblotted with anti-T7 and anti-HA antibodies.  (C) HeLa cells were transiently transfected with plasmids encoding HA-TAp73α, HA-ΔNp73α (lacking the TAD), T7-p73-DDα (lacking the TAD and DBD), HA-TAp73γ (lacking the SAM domain) or HA-TAp73α (L371P; lacking a functional OD).  10% of the whole cell extracts was used for inputs, while the remainder was immunoprecipitated with an anti-HA (12CA5) antibody (lanes 6, 7, 9 and 10) or anti-p73 (ER-15) antibody (lane 8) and immunoblotted with anti-T7 and anti-HA antibodies.  (Note that binding in lane 9 was observed at darker exposures.)
3.3.3. SATB2 affects p73 induction of target genes

To ask whether SATB2 affects the transcriptional activity of p73, semi-quantitative RT-PCR was used to determine the effect of SATB2 on the induction of p73-target gene transcript levels. Saos-2 cells were transiently transfected with plasmids encoding T7-SATB2 and either HA-TAp73α or HA-TAp73β. Total RNA was isolated and RT-PCR was performed using primers specific for PUMA, a pro-apoptotic gene, and p21, a cell cycle gene (Figure 3.7A and B, respectively). Densitometric analyses of PUMA and p21 mRNA levels were normalized to β-actin (internal control) and expressed as fold change. Increased levels of both PUMA and p21 induction were observed when SATB2 and TAp73α were co-expressed (lane 4), while SATB2 and TAp73β co-expression led to decreased levels (lane 6). Thus, when overexpressed, SATB2 was shown to be able to enhance TAp73α-mediated induction of PUMA and p21 transcripts. Conversely, SATB2 was shown to inhibit TAp73β-mediated induction of those target genes.
Figure 3.7

A.

![Graph showing fold change with HA-TAp73α, HA-TAp73β, and T7-SATB2 conditions.]

B.

![Graph showing fold change with HA-TAp73α, HA-TAp73β, and T7-SATB2 conditions.]

Legend:
- HA-TAp73α
- HA-TAp73β
- T7-SATB2
- PUMA
- β-actin
- p21
- β-actin
Figure 3.7. SATB2 affects TAp73 induction of target genes. Saos-2 cells were transiently transfected with plasmids encoding T7-SATB2 and HA-TAp73α or HA-TAp73β. Total RNA was isolated and RT-PCR was performed using primers specific for (A) PUMA or (B) p21 and β-actin (internal control). Densitometric analyses of PUMA and p21 mRNA levels were normalized to β-actin and expressed as fold change. A representative experiment (mean ± SD) of two to three independent experiments is shown.
3.3.4. SATB2 stabilizes p73α

When validating the interaction between p73 and SATB2, I detected higher levels of TAp73α in lanes in which SATB2 was co-expressed. To ask whether increased SATB2 expression leads to higher levels of TAp73α, Saos-2 cells were transiently co-transfected with a constant amount of the plasmid encoding HA-TAp73α and increasing amounts of the plasmid encoding T7-SATB2. These cells were lysed, equivalent amounts of total cellular proteins were resolved by SDS-PAGE and immunoblotted with an anti-T7, anti-HA and anti-α-tubulin (loading control) antibody (Figure 3.8A). Interestingly, increasing TAp73α levels (lanes 2-5) were detected with increasing SATB2 co-expression (lanes 3-6). Even the lowest level of SATB2 co-expression dramatically increased TAp73α protein that was detected (compare lanes 2 and 3), thereby suggesting that SATB2 co-expression may lead to the stabilization of TAp73α. To determine whether the increased levels of TAp73α are a result of SATB2 binding, similar experiments were performed substituting T7-SATB2 with T7-SATB2ΔPDZ (Figure 3.8B). With increasing SATB2ΔPDZ expression (lanes 3-6), TAp73α levels increased (lanes 2-5). These results suggest that only the co-expression of SATB2, rather than the actual binding of SATB2, is sufficient to detect the increased TAp73α levels; however, binding may strengthen the observed stabilization effect. Since SATB2 is also able to interact with ΔNp73α, I examined whether SATB2 expression can also lead to increased levels of ΔNp73α. Similar experiments were carried out using a constant amount of the plasmid encoding HA-ΔNp73α (Figure 3.8C). Increasing ΔNp73α levels (lanes 2-5) were observed with increasing SATB2 co-expression (lanes 3-6), and suggest that SATB2 co-expression may lead to the stabilization of ΔNp73α, as well as TAp73α.
In order to determine whether the increased levels of p73α detected were due to enhanced protein stabilization, cycloheximide, which inhibits protein synthesis, was used to examine protein half-life. Saos-2 cells were transiently transfected with plasmids encoding HA-TAp73α and T7-SATB2. Approximately 48 h post-transfection, cells were treated with cycloheximide and harvested at 0, 3, 6 and 9 h. These cells were then lysed, equivalent amounts of total cellular proteins were resolved by SDS-PAGE and immunoblotted with anti-T7, anti-HA and anti-α-tubulin (loading control) antibodies (Figure 3.8D). Densitometric analysis of TAp73α protein levels was normalized to α-tubulin and expressed as percent change (Figure 3.8E). At the 3-h time point, the levels of TAp73α decreased to 48% (in comparison to time 0), thereby indicating that the half-life of TAp73α is approximately 3 h. With SATB2 co-expression, the levels of TAp73α decreased to 63%. At the 6-h time point, TAp73α levels decreased to 31%, and 58% with SATB2 co-expression. These findings are also supported by pulse chase experiments (data not shown). Taken together, these data suggest that SATB2 co-expression prolongs the half-life of TAp73α. Thus, SATB2 expression leads to the stabilization of TAp73α.
Figure 3.8

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Figure 3.8

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![Graph showing changes over time](image)

E.  

![Graph showing percentage change over time](image)

- TAp73α
- TAp73α (+ SATB2)
**Figure 3.8. SATB2 stabilizes p73α.** (A) Saos-2 cells were transiently transfected with the same amount of the plasmid encoding HA-TAp73α and increasing amounts of the plasmid encoding T7-SATB2. Equivalent amounts of total cellular proteins were resolved by SDS-PAGE and immunoblotted with anti-T7, anti-HA and anti-α-tubulin (loading control) antibodies. (B) Saos-2 cells were transiently transfected with the same amount of the plasmid encoding HA-TAp73α and increasing amounts of the plasmid encoding T7-SATB2ΔPDZ. Equivalent amounts of total cellular proteins were resolved by SDS-PAGE and immunoblotted with anti-T7, anti-HA and anti-α-tubulin (loading control) antibodies. (C) Saos-2 cells were transiently transfected with the same amount of the plasmid encoding HA-ΔNp73α and increasing amounts of the plasmid encoding T7-SATB2. Equivalent amounts of total cellular proteins were resolved by SDS-PAGE and immunoblotted with anti-T7, anti-HA and anti-α-tubulin (loading control) antibodies. (D) Saos-2 cells were transiently transfected with plasmids encoding HA-TAp73α and T7-SATB2. Approximately 48 h post-transfection, cells were treated with cycloheximide and harvested at 0, 3, 6 and 9 h. Equivalent amounts of total cellular proteins were resolved by SDS-PAGE and immunoblotted with anti-T7, anti-HA and anti-α-tubulin (loading control) antibodies. (E) Densitometric analysis of TAp73α protein levels was normalized to α-tubulin (loading control) and expressed as percent change. A representative experiment of two independent experiments is shown.
3.3.5. SATB2 decreases p73β levels

In contrast to increased p73α detected when performing p73α and SATB2 co-IP experiments, I detected decreased p73β levels when SATB2 was co-expressed. In order to ask whether SATB2 affects p73β levels, Saos-2 cells were transiently co-transfected with the same amount of the plasmid encoding HA-TAp73β and increasing amounts of the plasmid encoding T7-SATB2. These cells were lysed, equivalent amounts of total cellular proteins were resolved by SDS-PAGE and immunoblotted with anti-T7, anti-HA and anti-α-tubulin (loading control) antibodies (Figure 3.9A). Interestingly, decreasing TAp73β protein (lanes 2-5) was detected with increasing amounts of co-expressed SATB2 (lanes 3-6), thereby suggesting that SATB2 co-expression may lead to the destabilization of TAp73β. Similar experiments were carried out using a constant amount of the plasmid encoding ΔNp73β (Figure 3.9B). Decreasing ΔNp73β levels were also observed with increasing SATB2 co-expression, and suggests that SATB2 co-expression may lead to the destabilization of ΔNp73β as well as TAp73β.

Like p73β, SATB2 does not bind to p53 (Figure 3.3). In order to determine whether SATB2 similarly affects p53 levels, Saos-2 cells were transiently co-transfected with the same amount of the plasmid encoding HA-p53 and increasing amounts of the plasmid encoding T7-SATB2 (Figure 3.9C). Decreased p53 levels were detected with SATB2 co-expression. Thus, like both TA and ΔNp73β, SATB2 modulates the levels of p53.

In order to examine whether SATB2 affects the levels of endogenous TAp73β under physiological conditions, SCC9 HNSCC cells that express TAp73β were used and SATB2 was expressed using adenoviral infection since SCC9 cells are very difficult to transfect. SCC9 cells were infected with Ad-SATB2 and Ad-GFP (control). After these cells were lysed, the proteins
were resolved by SDS-PAGE and immunoblotted with anti-SATB2, anti-p73 and anti-vinculin (loading control) antibodies (Figure 3.9D). Endogenous TAp73β levels decreased in the presence of SATB2 overexpression (compare lanes 2 and 3). Therefore, this experiment supports a role for SATB2 in modulating endogenous p73β levels in tumour cells.

In order to determine whether the decreased levels of p73β detected were due to protein destabilization, cycloheximide was used to examine protein half-life. Saos-2 cells were transiently transfected with plasmids encoding HA-TAp73β and T7-SATB2. Approximately 48 h post-transfection, cells were treated with cycloheximide and harvested at 0, 4, 8, 12 and 24 h. These cells were then lysed, equivalent amounts of total cellular proteins were resolved by SDS-PAGE and immunoblotted with anti-T7, anti-HA and anti-α-tubulin (loading control) antibodies (Figure 3.9E). Densitometric analysis of TAp73β protein levels was normalized to α-tubulin and expressed as percent change (Figure 3.9F). TAp73β levels initially decreased similarly with or without SATB2 co-expression, but at the 12-h time point, the levels of TAp73β decreased to 30%, and 5% with SATB2 co-expression. Since the levels of TAp73β were not different in the absence or presence of SATB2 at earlier time points, additional experiments are needed to further clarify whether SATB2 destabilizes TAp73β. Taken together, these data suggest that SATB2 expression decreases TAp73β levels and may lead to the destabilization of TAp73β.

Since the protein stability of p73 is affected by SATB2, and p73 sumoylation and ubiquitination have been reported to be important mechanisms by which p73 is degraded (Minty et al., 2000; Munarriz et al., 2004; Rossi et al., 2005), we hypothesized that proteasomal degradation may play a role in the destabilization of p73β by SATB2. To ask whether ubiquitin-mediated proteolysis may be involved, Saos-2 cells were transiently transfected with plasmids
encoding HA-TAp73β and T7-SATB2. Approximately 48 h post-transfection, the proteasome inhibitor MG-132 was then added. After these cells were lysed, the proteins were resolved by SDS-PAGE and immunoblotted with anti-T7, anti-HA, and anti-vinculin (loading control) antibodies (Figure 3.9G). As expected, in the absence of MG-132, TAp73β decreases with SATB2 co-expression (compare lanes 1 and 3). The decrease in TAp73β levels seen with SATB2 co-expression, however, is rescued by the addition of MG-132 (compare lanes 3 and 4). Therefore, the proteasome appears to play a role in the modulation of TAp73β levels by SATB2.
Figure 3.9

A. HA-TAp73β
   T7-SATB2
   T7 IB
   HA IB
   Tubulin IB

B. ΔNp73β
   T7-SATB2
   T7 IB
   p73 IB
   Tubulin IB

C. HA-p53
   T7-SATB2
   T7 IB
   HA IB
   Tubulin IB

D. Ad-GFP
   Ad-SATB2
   SATB2 IB
   p73 IB
   Vinculin IB
Figure 3.9

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T7 IB

HA IB

Tubulin IB

F.

% Change

TAp73β

TAp73β (+ SATB2)

Time (h)

G.

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T7 IB

HA IB

Vinculin IB
Figure 3.9. SATB2 decreases p73β levels. (A-C) Saos-2 cells were transiently transfected with a constant amount of the plasmid encoding (A) HA-TAp73β, (B) ΔNp73β or (C) HA-p53 and increasing amounts of the plasmid encoding T7-SATB2. Equivalent amounts of total cellular proteins were resolved by SDS-PAGE and immunoblotted with anti-T7, anti-HA or anti-p73 (GC-15), and anti-α-tubulin (loading control) antibodies. (D) SCC9 cells were infected with Ad-SATB2 and Ad-GFP (control). After these cells were lysed, the proteins were resolved by SDS-PAGE and immunoblotted with anti-SATB2, anti-p73 and anti-vinculin (loading control) antibodies. (E) Saos-2 cells were transiently transfected with plasmids encoding HA-TAp73β and T7-SATB2. Approximately 48 h post-transfection, cells were treated with cycloheximide and harvested at 0, 4, 8, 12, and 24 h. Equivalent amounts of total cellular proteins were resolved by SDS-PAGE and immunoblotted with anti-T7, anti-HA and anti-α-tubulin (loading control) antibodies. (F) Densitometric analysis of TAp73β protein levels was normalized to α-tubulin (loading control) and expressed as percent change. A representative experiment of two independent experiments is shown. (G) Saos-2 cells were transiently transfected with plasmids encoding HA-TAp73β and T7-SATB2. Approximately 48 h post-transfection, the proteasome inhibitor MG-132 was then added. After these cells were lysed, the proteins were resolved by SDS-PAGE and immunoblotted with anti-T7, anti-HA, and anti-vinculin (loading control) antibodies.
3.4. Discussion

Although numerous studies over the past ten years have focused on the p53 family, it is still not completely understood how p53, p63 and p73, as well as all their isoforms generated by both alternative splicing and differential promoter utilization, are differentially regulated. In comparison to p53 and p63, p73, by far, has the most structural complexity with the largest combination of N- and C-terminal isoforms. Several proteins have been identified that regulate the p53 family members and their isoforms in different ways. For example, MDM2 interacts with both p53 and p73, but results in proteasomal degradation only for p53 (Haupt et al., 1997; Honda et al., 1997; Kubbutat et al., 1997) and stabilization for p73 (Balint et al., 1999; Dobbelstein et al., 1999; Zeng et al., 1999). ITCH ubiquitinates only certain isoforms of p73 (Rossi et al., 2005), while PIAS1 sumoylates only the α variant of p73 (Munarriz et al., 2004). It is important, therefore, to uncover novel p53 family regulators in order to advance our understanding of this important family of transcription factors.

Here, I have identified SATB2 as a differential regulator of the p53 family—specifically, p73. I found that SATB2 interacted with p73α, and its co-expression led to increased TAp73α-mediated induction of target genes and the stabilization of TAp73α expression. Interestingly, SATB2 had the opposite effect on p73β despite the fact that I was unable to demonstrate an interaction between them. SATB2 co-expression resulted in inhibition of TAp73β-mediated induction of target genes and decreased TAp73β levels, which was linked to the proteasome pathway. In addition, although I determined that the PDZ-like domain of SATB2 is required for binding to p73 (Figure 3.6B), I was unable to narrow down the region of the C-terminus of p73 that was responsible for binding to SATB2. A common sequence shared only by p73α and γ (the
C-terminal isoforms to which SATB2 binds), but not p73β or δ, has not been identified except for the extreme C-terminus where the last four amino acids are similar between p73α and γ. Since PDZ domains bind to the C-terminal tail of proteins (Sheng and Sala, 2001) and SATB2 contains a PDZ-like domain that is required for binding to p73, perhaps binding occurs in the extreme C-terminal region of p73. The tertiary structure of p73 may also be important for binding given that SATB2 binding was specific for certain p73 C-terminal isoforms.

Moreover, since SATB2 had an effect on the activity and levels of TAp73β (Figure 3.7 and Figure 3.9, respectively) and SATB2ΔPDZ modulated TAp73α levels (Figure 3.8B), the effects of SATB2 on p73 may occur independent of binding. Given this, it would be of interest to examine whether a physical interaction is necessary for SATB2 to enhance the transcriptional activity of TAp73α. The semi-quantitative RT-PCR experiments could be repeated using TAp73α co-expressed with SATB2ΔPDZ in order to determine whether SATB2ΔPDZ, like wild-type SATB2, also enhances the TAp73α-mediated induction of PUMA and/or p21, thereby suggesting that SATB2 binding is not required to alter the transcriptional activity of TAp73. These observations also suggest that SATB2 may exert its effects on p73 by recruiting as-yet unidentified transcription factors for co-activation or co-repression and/or proteins involved in p73 stability and degradation. This is feasible since SATB2 has already been shown to enhance the transcriptional activity of the OB differentiation regulators RUNX2 and ATF4, rather than directly binding to the promoters of their target genes (Dobreva et al., 2006). In the case of p73β, SATB2 may engage ligases that specifically degrade p73β, or promote the shuttling of p73β into an inaccessible subcellular compartment, which may include the nuclear matrix. Thus, SATB2-mediated effects on p73β localization to a subcellular compartment that is not soluble
with our lysis conditions may account for the decreased detection of p73β protein on IBs in the presence of SATB2 co-expression and the inability to detect the effects of SATB2 expression in p73β half-life assays. Nevertheless, SATB2 appears to be involved in the regulation of p73 stability.

Since the primary experimental strategy used to study the interaction between p73 and SATB2 involved epitope-tagged proteins expressed in cultured cells, there are some major pitfalls associated with epitope tagging. Firstly, epitope-tagged proteins are expressed at higher than normal levels in cells. Plasmids encoding these epitope-tagged genes must also be introduced into cells by using transfection reagents, which can lead to issues arising from transfection efficiency and cytotoxicity. Moreover, epitope tagging may alter protein structure, function and subcellular localization. In the characterization of the p73-SATB2 interaction, epitope tagging was a beneficial experimental strategy since p73 is normally expressed at low levels and because of the lack of high-affinity antibodies to detect p73. In addition, SATB2 had yet to be identified when we first began studying this protein, and SATB2-specific antibodies were unavailable at that time. Hence, epitope tagging allowed us to initially study and characterize the interaction between p73 and SATB2.

The importance of the p53 family in cancer and development has been illustrated in a multitude of studies. Understanding the p73 network is important since, in tumours with mutated or inactivated p53, TAp73 is able to induce apoptosis in place of p53 by activating the promoters of numerous p53-family-target genes in response to DNA-damaging agents, such as chemotherapeutic drugs (Jost et al., 1997; Kaghad et al., 1997; Ishida et al., 2000; Harms et al., 2004). Since SATB2 has different effects on p73 C-terminal isoforms, the net effect of SATB2 expression may depend on the p73 isoform(s) expressed in a particular cell. Because SATB2 is
able to stabilize TAp73α levels and increase its induction of target genes involved in apoptosis or cell cycle arrest, increased SATB2 expression may be employed in cancers with mutated or inactivated p53 that also express TAp73α to enhance the tumour-suppressor-like ability of TAp73α. Similarly, because SATB2 is also able to modulate p73β levels and inhibit TAp73β-mediated induction of target genes, increased SATB2 expression may likewise be used in tumours that express ∆Np73β to diminish the levels of ∆Np73β and to repress the inhibitory effect of ∆Np73β on TAp73/p53. In contrast, in tumours that express TAp73β or ∆Np73α, decreased SATB2 expression in these tumours would result in increased TAp73β levels (apoptosis) or decreased ∆Np73α levels (survival), respectively. Therefore, I propose a model in which SATB2 differentially regulates the C-terminal isoforms of p73 to promote cell death or cell survival (Figure 3.10). For example, IMR-5 neuroblastoma cells, which express high levels of ∆Np73α, low levels of TAp73β and moderate levels of SATB2 (see Table 4.1), could be used to address this model experimentally. If SATB2 was knocked down in these cells using RNA interference (RNAi), we would expect the levels of ∆Np73α to decrease and TAp73β to increase, thereby pushing the balance between TA versus ∆N towards cell death. The expression levels of downstream target genes could also be examined either by RT-PCR or immunoblotting in order to determine whether there is an increase in the levels of pro-apoptotic genes/proteins, such as PUMA. It is thus very important for future studies focusing on p73 expression in cancers to differentiate between specific isoforms of p73—not only the TA and ∆N variants, but the C-terminal isoforms as well. Previous studies by our laboratory and others have shown that the levels of α isoforms detected in tumour cell lines are often higher than β. It remains to be determined whether SATB2 expression may influence the expression pattern of α versus β.
isoforms in tumours. Therefore, the dual functionality of SATB2 lends this protein to diverse roles in cancer, and its ability to differentially regulate p73 make it suitable as a target for future drug development.

Other future areas of study include luciferase and/or chromatin IP (ChIP) assays to further investigate the role of SATB2 on p73-mediated transcription of target genes. These experiments would enable us to determine whether SATB2 recruits p73 to the binding sites and acts as a co-activator of p73, or whether SATB2 binds to the promoters themselves. In HNSCC, our laboratory recently showed that SATB2 interacts with ΔNp63α and enhances its binding to p53-target gene promoters, such as PUMA and p21, thereby promoting survival (Chung et al., 2010). Interestingly, in contrast to its effects on p73α levels, SATB2 did not affect the stability of ΔNp63α. Similarly, I presume that SATB2 may act as a co-activator of p73 by recruiting p73 to the promoters of its target genes. In addition, I demonstrated that the decreased levels of TAp73β mediated by SATB2 is rescued by the addition of a proteasomal inhibitor (Figure 3.9G). This finding suggests that SATB2 promotes the proteasomal-dependent degradation of TAp73β, and may accomplish this function by recruiting ligases. Future studies will determine whether known p73 ligases, such as ITCH or PIAS1, or unidentified ligases promote the proteasomal degradation of p73 in concert with SATB2. Although a pilot study examining the detergent-insoluble fraction from CHX experiments with p73α and SATB2 co-expression did not show any obvious differences compared to the detergent-soluble fraction (data not shown), future experiments with p73β and SATB2 co-expression should be performed to determine whether p73β becomes insoluble when SATB2 is present. Moreover, ΔNp73β was shown to be expressed in peripheral sympathetic and cortical neurons (Pozniak et al., 2000; Pozniak et al., 2002) and SATB2 in post-mitotic, differentiating cortical neurons (Britanova et al., 2005;
Szemes et al., 2006). Since both SATB2 and p73 have been independently involved in neuronal development, their interaction may play a role in the developing nervous system. Therefore, these studies may ultimately help to elucidate the unique regulatory pathways for p73 and SATB2 in cancer and development.
Figure 3.10. Proposed differential regulation of p73 C-terminal isoforms by SATB2. SATB2 expression leads to increased levels of TAp73α and ΔNp73α, but decreased levels of TAp73β and ΔNp73β. ΔNp73 is also able to inhibit TAp73. Thus, SATB2 expression can lead to both cell death and cell survival, depending on the specific p73 C-terminal isoform expression profile in a given cell.
Chapter 4:
Role of SATB2 in osteosarcoma and invasion

The work presented in this chapter was completed by me, except for parts of Table 4.1 by Lynn Cheng, Figure 4.2A and B by Ivan Pasic and Dr. David Malkin, and Figure 4.2C by Dr. Paul Thorner.

4.1. Introduction

OS is the most common type of bone cancer, and although it is the 8th most common paediatric cancer, OS is the 2nd leading cause of paediatric cancer death (Ottaviani and Jaffe, 2010; Kim et al., 2010; Dass et al., 2006). The use of multi-agent chemotherapy and improved surgical techniques have now increased the overall survival rates to 65-70% (Pizzo and Poplack, 2006). However, 20-25% of OS patients present with metastases, most commonly in the lungs (Bacci et al., 2008), and these patients have a worse prognosis with much lower overall survival rates of about 10-20% (Wu et al., 2009). The discovery of clinical markers to aid in the identification and prognosis of OS would be potentially beneficial.

OS primarily occurs near the area of most active bone growth in childhood, suggesting an association between OB activity and tumour development (Arndt and Crist, 1999; Kansara and Thomas, 2007). The etiologies of OS remain elusive; however, radiation exposure, specific cytogenetic abnormalities, the p53 and RB1 tumour suppressor pathways, and RUNX2 (a regulator of OB growth and differentiation) expression and activity have been implicated (reviewed in Ta et al., 2009). OB-specific deletion of both p53 and RB1 were sufficient for
developing a mouse model that resembles human OS and included metastatic disease (Walkley et al., 2008; Berman et al., 2008). Interestingly, these studies revealed that unlike mice with \( p53 \) deletions that developed sarcomas, mice with homozygous or heterozygous deletions of \( RB1 \) did not develop OS, thereby indicating that \( p53 \) is important in the initiation of OS development and \( RB1 \) acts in synergy with \( p53 \) to potentiate p53 effects.

Although the mechanisms underlying OS metastasis are poorly understood, several genes have been implicated, including \( MMPs \), \( TIMPs \) and \( RUNX2 \). MMPs, proteolytic enzymes that function in protein degradation, were found to be overexpressed in OS cell lines and this overexpression was associated with metastasis (Bjornland et al., 2005). Conflicting roles have been reported for both TIMP and RUNX2. TIMP1, a natural inhibitor of MMPs, has been associated with OS metastasis and cell survival and growth (Ferrari et al., 2004; Hornebeck et al., 2005). Increased RUNX2 expression has been associated with poor patient survival and tumour metastasis in OS (Won et al., 2009) and RUNX2 was reported to repress the cell cycle gene \( p21 \) in OS cells (Westendorf et al., 2002). In contrast, RUNX2 was also reported to induce the pro-apoptotic gene \( Bax \) in OS cells (Eliseev et al., 2008).

SATB2, a nuclear MAR-binding protein and transcriptional regulator, has been linked with both OB differentiation and RUNX2. SATB2 controls OB differentiation by regulating OB-specific genes, such as \( Bsp \), \( Ocn \) and \( Hoxa2 \), and by enhancing the transcriptional activity of OB differentiation regulators, such as RUNX2 and ATF4 (Dobreva et al., 2006). In addition, significant bone defects were observed in \( SATB2^{-/-} \) mice. Although these studies support a role for SATB2 in OBs, the cell of origin for OS, a role for SATB2 in OS has not been reported. In other solid tumours including sarcomas, several studies have implicated not only the SATB family in tumourigenesis and metastasis, but also the activation of other pathways. Breast
tumour growth and metastasis were promoted by SATB1. SATB1, but not SATB2, was expressed only in aggressive, metastatic breast cancer cell lines and also altered the gene expression profile, and specifically genes involved in invasion and metastasis, in breast cancer cells (Han et al., 2008). Our laboratory has also recently shown that SATB2 mediates chemoresistance in HNSCC and its expression is highest in advanced-stage HNSCC tumours (Chung et al., 2010). In addition, SATB2 has been shown to be upregulated in canine OS as detected by a gene expression array (Wensman et al., 2009). Conflicting data was reported in another breast cancer study, in which SATB2 was associated with increasing tumour grade and poorer overall survival (Patani et al., 2009). Moreover, downregulation of SATB2 was associated with metastasis and poor prognosis in colorectal cancer (Wang et al., 2009).

Since several studies have linked the expression of the SATB family proteins to cancer development and progression and SATB2 plays an important role in OBs during development, we hypothesized that SATB2 may be involved in OS and, in particular, possibly OS invasion and metastasis. Here, I report that SATB2 expression was highly elevated in both OS cell lines and OS tumour samples in comparison to normal, non-transformed OBs. SATB2 expression was also detected in the majority of OS samples but not in other sarcomas and thus, may assist in distinguishing OS from other sarcomas clinically. Moreover, OS cells in which SATB2 had been knocked down had diminished invasive and migratory capabilities. Thus, these observations indicate that SATB2 plays an important role in OS invasion.
4.2. Methods

4.2.1. Cell culture
Saos-2 and U2OS human OS, and HaCat human keratinocyte cell lines were obtained from ATCC (Rockville, MD). HOS, KHOS and MNNG human OS cell lines were generously provided by Dr. David Malkin. Primary human OBs were obtained from Promocell (Heidelberg, Germany). Primary human OBs were cultured in complete Osteoblast Growth Medium (Promocell) and all other cells were cultured in DMEM (Gibco; Grand Island, NY) supplemented with 10% FBS (Hyclone; Logan, UT). All cells were maintained at 37°C in a humidified 5% CO2 incubator.

4.2.2. Quantitative RT-PCR
OS samples (n = 43) were obtained, and mRNA was isolated and analyzed by quantitative RT-PCR (qRT-PCR) as described previously (Pasic et al., 2010) in collaboration with Ivan Pasic and Dr. David Malkin. Levels of \( SATB2 \) expression were normalized to primary OBs, and a 1.5-fold change in expression levels was used as the cut-off. Primer sequences are as follows: \( SATB2 \) 5’-TCC TTA TAT TCA GCC ACG TCC A-3’ and 5’-AGG AAG CCA TCC ACA CTC TTT C-3’, and \( RUNX2 \) 5’-AGA GTC ATT TAA GCC TGC AA-3’ and 5’-AGA ACT TGT ACC CTC TGT TGT AA-3’.

4.2.3. Immunohistochemistry
OS samples were obtained in accordance with the research ethics guidelines of The Hospital for Sick Children (Toronto, ON). Immunohistochemistry (IHC) was performed on histologic
sections of formalin-fixed, paraffin-embedded tumour biopsy samples in collaboration with Dr. Paul Thorner. These tumour samples were immunostained with anti-SATB2-CT antibody and scored for SATB2 positivity or negativity.

### 4.2.4. SATB2 stable knockdown cells

Viral work was performed in accordance with the safety guidelines of The Hospital for Sick Children (Toronto, ON) and lentivirus constructs and virus were produced as described previously (Moffat et al., 2006). KHOS cells were seeded and the next day, they were washed with PBS and then infected with a 2-mL total volume made up of 1 mL of lentivirus and 1 mL of DMEM supplemented with 10% FBS containing 8 μg/mL polybrene (hexadimethrine bromide; Sigma-Aldrich; Oakville, ON). Lentivirus expressing two different short hairpin RNA (shRNA) sequences directed against different regions of SATB2 (shSATB2-1 and shSATB2-2) were used to knock down SATB2. Lentivirus expressing shGFP was used as a negative control. The lentivirus shRNA sequences were as follows: shSATB2-1 5’-ccg gCG ACA TGC TAC AAG ATG TCT Act cga gTA GAC ATC TTG TAG CAT GTC Gtt ttt-3’ and 5’-aaa aaC GAC ATG CTA CAA GAT GTC TAc tcg agT AGA CAT CTT GTA GCA TGT CGc cgg-3’; shSATB2-2 5’-ccg gGC CAT GCA GAA TTT CCT CAA Tct cga gAT TGA GGA AAT TCT GCA TGG Ctt ttt-3’ and 5’-aaa aaG CCA TGC AGA ATT TCC TCA ATc tcg agA TTG AGG AAA TTC TGC ATG GCT ttt t-3’; and shGFP 5’-ccg gGG TGC GCT CCT GGA CGT AGC Cct cga gGG CTA CGT CCA GGA GCG CAC Ctt ttt-3’ and 5’-aaa aaG GTG CGC TCC TGG AGC TAG CCc tcg agG GCT ACG TCC AGG AGC GCA CCc cgg-3’. 24 h-post infection, cells were washed at least 5 times with PBS and were selected in DMEM supplemented with 10% FBS and 1 μg/mL puromycin (Sigma-Aldrich; Oakville, ON). Puromycin-resistant clones were isolated and
screened for SATB2 knockdown by immunoblotting. Cells stably expressing shSATB2-1, shSATB2-2 and shGFP were designated KHOS-shSATB2-1, KHOS-shSATB2-2 and KHOS-shGFP, respectively. Knockdown efficiency was monitored by IB and evaluated at the time of the described functional experiments.

4.2.5. Immunoblotting

Cells in 10-cm tissue culture plates were scraped, centrifuged, and washed in cold PBS. These cells were then lysed in EBC lysis buffer supplemented with complete protease inhibitors (Roche; Indianapolis, IN) at 4°C for 30 min. Samples were centrifuged at 4°C for 10 min to remove cell debris. The Bradford method (Bio-Rad; Hercules, CA) was used to determine protein concentrations. Samples were boiled in sample buffer, proteins were resolved by SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad; Hercules, CA). Membranes were blocked in TBS supplemented with 5% (wt/vol) powdered milk for 1 h at room temperature and incubated with primary antibody at 4°C overnight. Membranes were then washed five times with TBST for 5 min at room temperature and incubated with horseradish peroxidase-conjugated goat anti-mouse or -rabbit secondary antibody (Pierce Biotechnology; Rockford, IL) in TBS supplemented with 2% (wt/vol) powdered milk for 1 h at room temperature. Membranes were again washed five times with TBST and bound secondary antibody was detected by enhanced chemiluminescence with Supersignal (Pierce Biotechnology; Rockford, IL).

4.2.6. Invasion/migration assay

3 μl of Matrigel basement membrane matrix (BD Biosciences; Mississauga, ON) was diluted with 47 μl of serum-free DMEM (Gibco) for each required cell culture insert consisting of a
membrane with 8-μm pores (BD Biosciences). Inserts layered with diluted Matrigel were used to measure cell invasion, or without Matrigel to measure cell migration. 2×10⁴ KHOS-shSATB2 cells in low-serum media (DMEM supplemented with 0.1% FBS) were added to the inserts in triplicates. DMEM supplemented with 10% FBS were added to the wells to act as a chemoattractant. After incubation for approximately 24 h, non-invading cells were removed and cells on the undersides of the membranes were fixed and stained with 1% crystal violet in 20% methanol. Pictures of five fields of view under the microscope were taken and pixel counts associated with cell numbers were measured using Scion Image (http://www.scioncorp.com/), normalized to the control, and expressed as fold change.

4.2.7. Scratch assay

KHOS-shSATB2 cells were grown to a confluent monolayer in 6-cm plates. A line was scratched through the cells in 3-5 replicates and low-serum media (DMEM supplemented with 0.5% FBS) were added to the plates. Images of each scratch were captured at 0, 6, 12 and 24 h, and the wound widths were measured, normalized to the control, and expressed as percent recovery.

4.2.8. MTT assay

KHOS-shSATB2 cells were seeded in low-serum media (DMEM supplemented with 0.1% FBS) in quadruplicates. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Roche Applied Science; Branford, CT) was performed according to the manufacturer’s instructions. After incubation for approximately 24 h, the absorbance was measured by a spectrophotometer, normalized to the control, and expressed as fold change.
4.2.9. Expression microarray analysis

Total RNA was extracted with TRIzol Reagent (Invitrogen; Carlsbad, CA) from KHOS-shSATB2 cells. Analysis of GeneChip Human Gene 1.0 ST Arrays (Affymetrix; Santa Clara, CA) was performed by the Microarray Analysis and Gene Expression Facility at The Hospital for Sick Children. Ongoing data analysis is being performed in collaboration with Dr. Margaret Pienkowska and Dr. David Malkin using Ingenuity software (Ingenuity Systems; Redwood City, CA). A 1.5-fold change in expression levels was used as the cut-off.
4.3. Results

4.3.1. SATB2 expressed in OS cell lines

We observed high levels of SATB2 expression in the Saos-2 OS cell line and examined levels of SATB2 expression in a variety of cancer cell lines (Table 4.1). In order to determine whether SATB2 levels were generally high in OS, various human OS cell lines were screened in comparison to control primary OBs since OBs are believed to be the cell of origin for OS. Cells were lysed, and proteins were resolved by SDS-PAGE and immunoblotted with anti-SATB2-CT and anti-vinculin antibodies (Figure 4.1). HaCat cells were used as a negative control (lane 7) and were also infected with an adenoviral vector encoding SATB2 for use as a positive control (lane 8). High levels of SATB2 were detected in all of the OS cell lines (lanes 2-6), especially in comparison to the OB control (lane 1). Interestingly, the levels were highest in Saos-2 cells (lane 6), which are $p53^{-/-}$, and lowest in U2OS cells (lane 5), which express wild-type p53. In contrast, SATB1 levels were not readily detected in these OS cell lines (data not shown).
Table 4.1. Qualitative analysis of SATB2 protein expression in cancer cell lines.

150 μg of protein were resolved by SDS-PAGE and immunoblotted with anti-SATB2 antibody. SATB2 protein levels were estimated using Saos-2 as a reference point for high (+++), breast cancer as a negative control for undetectable expression (–).

<table>
<thead>
<tr>
<th>Cancer Type</th>
<th>Cell Line</th>
<th>SATB2</th>
</tr>
</thead>
<tbody>
<tr>
<td>OS</td>
<td>HOS</td>
<td>++</td>
</tr>
<tr>
<td>OS</td>
<td>MNNG</td>
<td>++</td>
</tr>
<tr>
<td>OS</td>
<td>Saos-2</td>
<td>+++</td>
</tr>
<tr>
<td>OS</td>
<td>U2OS</td>
<td>++</td>
</tr>
<tr>
<td>Breast Carcinoma</td>
<td>MCF7</td>
<td>–</td>
</tr>
<tr>
<td>Cervical Carcinoma</td>
<td>HeLa</td>
<td>–</td>
</tr>
<tr>
<td>Colorectal Carcinoma</td>
<td>HCT116 (p53+/+)</td>
<td>++</td>
</tr>
<tr>
<td>Colorectal Carcinoma</td>
<td>HCT116 (p53−/−)</td>
<td>++</td>
</tr>
<tr>
<td>Glioblastoma</td>
<td>M059J</td>
<td>–</td>
</tr>
<tr>
<td>Glioblastoma</td>
<td>M059K</td>
<td>–</td>
</tr>
<tr>
<td>HNSCC</td>
<td>SCC9</td>
<td>++</td>
</tr>
<tr>
<td>HNSCC</td>
<td>SCC17A</td>
<td>++</td>
</tr>
<tr>
<td>HNSCC</td>
<td>SCC25</td>
<td>–</td>
</tr>
<tr>
<td>Lung Carcinoma</td>
<td>H1299</td>
<td>++</td>
</tr>
<tr>
<td>Lung Carcinoma</td>
<td>H157</td>
<td>++</td>
</tr>
<tr>
<td>Lung Carcinoma</td>
<td>H2170</td>
<td>+</td>
</tr>
<tr>
<td>Lung Carcinoma</td>
<td>H520</td>
<td>+++</td>
</tr>
<tr>
<td>Lung Carcinoma</td>
<td>MGH7</td>
<td>+</td>
</tr>
<tr>
<td>Lung Carcinoma</td>
<td>SK-MES-1</td>
<td>+</td>
</tr>
<tr>
<td>Lung Carcinoma</td>
<td>SW900</td>
<td>+</td>
</tr>
<tr>
<td>Medulloblastoma</td>
<td>Daoy</td>
<td>+++</td>
</tr>
<tr>
<td>Medulloblastoma</td>
<td>ONS76</td>
<td>++</td>
</tr>
<tr>
<td>Medulloblastoma</td>
<td>UW228</td>
<td>++</td>
</tr>
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<td>Medulloblastoma</td>
<td>UW426</td>
<td>+</td>
</tr>
<tr>
<td>Neuroblastoma</td>
<td>IMR-5</td>
<td>++</td>
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<tr>
<td>Rhabdomyosarcoma</td>
<td>RD</td>
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</tr>
<tr>
<td>Rhabdomyosarcoma</td>
<td>Rh4</td>
<td>+</td>
</tr>
<tr>
<td>Rhabdomyosarcoma</td>
<td>Rh30</td>
<td>+</td>
</tr>
</tbody>
</table>

High (+++), moderate (++) and low (+) and undetectable (−) SATB2 protein expression
Figure 4.1. SATB2 expressed in OS cell lines. Cells were lysed, proteins were resolved by SDS-PAGE, and immunoblotted with anti-SATB2 and anti-vinculin antibodies. HaCat cells were used as a negative control and were also infected with an adenoviral vector encoding SATB2 for use as a positive control.
4.3.2. SATB2 expressed in OS tumours

To examine the mRNA expression of SATB2 in OS samples, qRT-PCR was performed in collaboration with Ivan Pasic in Dr. David Malkin’s laboratory. Levels of SATB2 expression were normalized to primary OBs (Figure 4.2A). Of 43 samples, 24 (56%) showed elevated levels of SATB2 mRNA in comparison with the OB control. Since elevated levels of RUNX2 expression have been reported in OS cells (Nathan et al., 2009) and RUNX2 interacts with SATB2 in OBs (Dobreva et al., 2006), we examined RUNX2 mRNA levels in these OS samples as a comparison (Figure 4.2B). Increased RUNX2 expression was observed in 35 samples (81%). Thus, more than half of the OS samples we examined had elevated levels of SATB2 mRNA expression and there was not a clear correlation with the expression of RUNX2, which is a SATB2-interacting protein.

In order to determine whether SATB2 protein is expressed in OS tumours, IHC was used to examine SATB2 expression in a tissue microarray of OS samples in collaboration with Dr. Paul Thorner. These tumour biopsy samples were immunostained with anti-SATB2 antibody and initially scored as strong diffuse, moderate diffuse, focal or negative SATB2 staining. Since the majority of SATB2-positive tumour samples showed strong diffuse staining, they were re-categorized as SATB2 positive (brown) or negative (Figure 4.2C, upper panel). Other sarcoma types, such as rhabdomyosarcomas, Ewing’s sarcomas and soft tissue sarcomas, were also stained and scored for SATB2 positivity or negativity (Figure 4.2C, lower panel). 32 of 43 (74%) OS samples stained positive for SATB2, while 79 of 81 (98%) non-OS sarcomas were SATB2 negative ($\chi^2 = 73.32$, df = 3, $p < 0.01$). Hence, SATB2 may be a potential clinical marker of OS and aid in distinguishing OS from other types of sarcomas.
Figure 4.2

A. SATB2

B. RUNX2
Figure 4.2. SATB2 expressed in OS tumours. (A-B) OS samples (n = 43) were obtained, processed and analyzed by qRT-PCR. Levels of (A) SATB2 or (B) RUNX2 mRNA expression (mean ± SD) were normalized to primary OBs. A 1.5-fold change in expression levels was used as the cut-off. (C) Upper panel: representative OS tumour biopsy samples were immunostained with anti-SATB2-CT and show either positive (brown) or negative SATB2 staining. Lower panel: several sarcoma types were immunostained with anti-SATB2-CT and scored for SATB2 positivity or negativity.
4.3.3. SATB2 involved in OS cell migration and invasion

Since the SATB2 homologue, SATB1, regulates genes involved in invasion and metastasis (Han et al., 2008), we hypothesized that in OS, SATB2 might regulate metastasis. To study the role of SATB2 in OS, I hypothesized that knockdown rather than overexpression of SATB2 would be more informative since high levels of SATB2 are present in OS cell lines. Therefore, KHOS cells were infected with lentiviral vectors encoding two shRNA sequences directed against different regions of SATB2 (shSATB2-1 and shSATB2-2) to obtain efficient knockdown. Cells stably expressing shSATB2-1, shSATB2-2 and control shGFP were designated KHOS-shSATB2-1, KHOS-shSATB2-2 and KHOS-shGFP, respectively. In order to determine their levels of SATB2 protein expression, aliquots of these cells were lysed, proteins were resolved by SDS-PAGE, and immunoblotted with anti-SATB2 and anti-vinculin antibodies (Figure 4.3A). SATB2 was readily detected in both KHOS and KHOS-shGFP cells (lanes 1 and 2), but was substantially reduced in KHOS-shSATB2-1 and KHOS-shSATB2-2 (lanes 3 and 4), thereby validating SATB2 knockdown in these cells.

To begin examining whether SATB2 plays a role in OS cell migration, I performed migration assays using cell inserts consisting of membranes with 8-μm pores. KHOS-shSATB2-1, KHOS-shSATB2-2 and KHOS-shGFP (control) cells in low-serum media (DMEM supplemented with 0.1% FBS) were first added to the inserts in triplicate. DMEM supplemented with 10% FBS was added to the wells to act as a chemoattractant. After incubation for approximately 24 h, non-migrating cells were removed and cells on the undersides of the membranes were fixed and stained. Pictures of five fields of view under the microscope were taken and pixel counts associated with cell numbers were measured, normalized to control, and expressed as fold change (Figure 4.3B). In comparison to control KHOS-shGFP cells, 50-70%
fewer KHOS-shSATB2 cells migrated, thereby indicating that these cells had a diminished capacity to migrate towards the chemoattractant without SATB2. Decreased migration was also observed in transient experiments using HOS cells (another OS cell line) that had been infected with lentivirus expressing shSATB2 for efficient knockdown of SATB2 (data not shown).

An alternative approach to examine a role for SATB2 in OS cell migration is the scratch assay. KHOS-shSATB2 and KHOS-shGFP (control) cells were grown to a confluent monolayer and a line was scratched through the cells. Low-serum media (DMEM supplemented with 0.5% FBS) were added to the plates in order to inhibit cell proliferation. Images were captured at various time points to show the cells migrating to close the scratch (Figure 4.3C). The wound widths were measured, normalized to control and expressed as percent recovery (Figure 4.3D). KHOS-shSATB2 cells took longer to close the scratch defect than control cells, adding further support for SATB2 in mediating cell migration.

To determine whether SATB2 modulates cell invasion in OS, I performed cell invasion assays in which cell inserts that consisted of Matrigel layered over membranes with 8-μm pores were used. Matrigel is a reconstituted, in vitro basement membrane and only invasive cells are able to digest the Matrigel and migrate through the membrane pores. KHOS-shSATB2-1, KHOS-shSATB2-2 and KHOS-shGFP (control) cells in low-serum media (DMEM supplemented with 0.1% FBS) were first added to the inserts in triplicate. DMEM supplemented with 10% FBS was added to the wells to act as a chemoattractant. After incubation for approximately 24 h, non-migrating cells were removed and cells on the undersides of the membranes were fixed and stained. Pictures of five fields of view under the microscope were taken and pixel counts associated with cell numbers were measured, normalized to control, and expressed as fold change (Figure 4.4). In comparison to control KHOS-shGFP cells, 75-85%
fewer KHOS-shSATB2 cells invaded through the Matrigel, and indicates that the invasive
capability of these cells with low SATB2 levels was reduced. In transient experiments using
HOS cells (another OS cell line) in which SATB2 had been knocked down, decreased invasion
was also observed (data not shown).

Since differences in the rate of cell growth may potentially account for the diminished
invasion and migration observed in OS cells lacking SATB2 expression versus control cells,
MTT assays were used to examine OS cell growth. KHOS-shSATB2 and KHOS-shGFP
(control) cells were seeded in quadruplicates in low-serum media (conditions used in the inserts
of the migration/invasion assays) for the MTT assays. After incubation for approximately 24 h,
the absorbance was measured, normalized to control, and expressed as fold change (Figure 4.5).
The cell growth of KHOS-shSATB2 cells was comparable to that of KHOS-shGFP cells, thereby
suggesting that the reduced migratory and invasive capabilities of OS cells with SATB2
knockdown could not be due to decreased cell growth. MTT assays were also performed using
media supplemented with 10% FBS media (conditions used in the wells of the
migration/invasion assays) and similar results were obtained (data not shown). Taken together,
our findings suggest that SATB2 promotes cell migration and invasion in OS, but does not have
a direct role in cell growth.
Figure 4.3

A.

KHOS
KHOS-shGFP
KHOS-shSATB2-1
KHOS-shSATB2-2

SATB2 IB
Vinculin IB

1 2 3 4

B.

Fold Change

KHOS-shGFP
KHOS-shSATB2-1
KHOS-shSATB2-2
Figure 4.3

C.

0 h | 6 h | 12 h | 24 h
---|---|---|---
KHOS-shGFP
KHOS-shSATB2-1
KHOS-shSATB2-2

D.

% Recovery vs Time (h)

- KHOS-shGFP
- KHOS-shSATB2-1
- KHOS-shSATB2-2
**Figure 4.3. SATB2 involved in OS cell migration.** (A) KHOS (parental), KHOS-shGFP (control), KHOS-shSATB2-1 and KHOS-shSATB2-2 were lysed, proteins were resolved by SDS-PAGE, and immunoblotted with anti-SATB2 and anti-vinculin antibodies. (B) KHOS-shSATB2 and KHOS-shGFP (control) cells in low-serum media (DMEM supplemented with 0.1% FBS) were first added to cell inserts consisting of membranes with 8-μm pores in triplicate. DMEM supplemented with 10% FBS was added to the wells to act as a chemoattractant. After incubation for approximately 24 h, non-migrating cells were removed and cells on the undersides of the membranes were fixed and stained with 1% crystal violet in 20% methanol. Pictures of five fields of view under the microscope were taken and pixel counts associated with cell numbers were measured, normalized to control, and expressed as fold change. A representative experiment (mean ± SD) of three independent experiments is shown. (C) KHOS-shSATB2 and KHOS-shGFP (control) cells were grown to a confluent monolayer and a line was scratched through the cells. Low-serum media (DMEM supplemented with 0.5% FBS) were added to the plates in order to inhibit cell proliferation. Images were captured at various time points to show the cells migrating to close the scratch. (D) The wound widths were measured, normalized to control and expressed as percent recovery. A representative experiment of two independent experiments is shown.
**Figure 4.4. SATB2 involved in OS cell invasion.** KHOS-shSATB2 and KHOS-shGFP (control) cells in low-serum media (DMEM supplemented with 0.1% FBS) were first added to cell inserts consisting of Matrigel layered over membranes with 8-μm pores in triplicate. DMEM supplemented with 10% FBS was added to the wells to act as a chemoattractant. After incubation for approximately 24 h, non-invading cells were removed and cells on the undersides of the membranes were fixed and stained with 1% crystal violet in 20% methanol. Pictures of five fields of view under the microscope were taken and pixel counts associated with cell numbers were measured, normalized to control, and expressed as fold change. A representative experiment (mean ± SD) of three independent experiments is shown.
Figure 4.5. OS cell growth unaffected in cells lacking SATB2. KHOS-shSATB2 and KHOS-shGFP (control) cells were seeded in low-serum media (DMEM supplemented with 0.1% FBS) in quadruplicates for a MTT assay. After incubation for approximately 24 h, the absorbance was measured, normalized to control, and expressed as fold change. A representative experiment (mean ± SD) of three independent experiments is shown.
4.3.4. Gene expression profile

Since SATB2 has been shown to promote migration and invasion of OS cells, an expression microarray analysis was performed in order to discover novel SATB2 target genes, including potential gene candidates associated with metastasis. Ongoing data analysis is being performed in collaboration with Dr. Margaret Pienkowska and Dr. David Malkin. A preliminary analysis revealed that many genes are differentially expressed in OS cells with SATB2 knockdown compared to control cells (Table 4.2). Therefore, the genes identified in this microarray will be validated and examined for their potential roles in invasion and metastasis.
Table 4.2. The most differentially expressed genes identified in a preliminary microarray analysis.

<table>
<thead>
<tr>
<th>Upregulated genes (fold change over KHOS-shGFP control)</th>
<th>KHOS-shSATB2-1</th>
<th>KHOS-shSATB2-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCL10</td>
<td>6.733</td>
<td>9.796 *</td>
</tr>
<tr>
<td>KRT8</td>
<td>6.366 *</td>
<td>4.645 *</td>
</tr>
<tr>
<td>FAM38B</td>
<td>6.000 *</td>
<td>4.183</td>
</tr>
<tr>
<td>EFEMP1</td>
<td>5.958 *</td>
<td>3.815 *</td>
</tr>
<tr>
<td>MMP16</td>
<td>5.853 *</td>
<td>3.572 *</td>
</tr>
<tr>
<td>CDH6</td>
<td>5.401 *</td>
<td>3.519</td>
</tr>
<tr>
<td>NNMT</td>
<td>5.229 *</td>
<td>3.504 *</td>
</tr>
<tr>
<td>GPR126</td>
<td>4.622 *</td>
<td>3.409</td>
</tr>
<tr>
<td>HBE1</td>
<td>4.092 *</td>
<td>3.149 *</td>
</tr>
<tr>
<td>LIMA1</td>
<td>3.881 *</td>
<td>3.125 *</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Downregulated genes (fold change over KHOS-shGFP control)</th>
<th>KHOS-shSATB2-1</th>
<th>KHOS-shSATB2-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>MUM1L1</td>
<td>-4.226 ^</td>
<td>-10.425 ^</td>
</tr>
<tr>
<td>ENPP1</td>
<td>-4.193 ^</td>
<td>-6.873</td>
</tr>
<tr>
<td>NOX4</td>
<td>-3.588 ^</td>
<td>-6.580 ^</td>
</tr>
<tr>
<td>ITGA8</td>
<td>-3.400 ^</td>
<td>-5.972 ^</td>
</tr>
<tr>
<td>MFAP2</td>
<td>-2.690 ^</td>
<td>-5.112</td>
</tr>
<tr>
<td>PLXNA2</td>
<td>-2.687 ^</td>
<td>-4.733</td>
</tr>
<tr>
<td>ESAM</td>
<td>-2.665 ^</td>
<td>-4.576 ^</td>
</tr>
<tr>
<td>PRRX1</td>
<td>-2.661 ^</td>
<td>-4.415 ^</td>
</tr>
<tr>
<td>PDE3A</td>
<td>-2.641 ^</td>
<td>-3.684 ^</td>
</tr>
<tr>
<td>ROBO4</td>
<td>-2.605 ^</td>
<td>-3.663 ^</td>
</tr>
</tbody>
</table>

Upregulated (*) and downregulated (^) genes in both KHOS-shSATB2-1 and 2 cells
4.4. Discussion

Although OS is the most common type of bone cancer and the 2nd leading cause of paediatric cancer death (Dass et al., 2006), the genes and pathways involved in OS metastasis are still not well understood; however, radiation exposure, cytogenetic abnormalities, the \textit{p53} and \textit{RB1} tumour suppressor pathways, and RUNX2 (a regulator of OB growth and differentiation) expression and activity have been implicated in a subset of OS (reviewed in Ta et al., 2009). Although some genes and pathways have been reported to play a role in OS metastasis, none have led to the identification of novel therapies.

In this report, I present the first evidence of the involvement of SATB2 in OS not only as a potential marker specific for OS, but also as a regulator in invasion. Elevated levels of SATB2 mRNA and protein were detected in the majority of the OS tumours examined. These findings are supported by another study in which \textit{SATB2} was found to be upregulated in canine mammary OS in a gene expression array (Wensman et al., 2009), but are in contrast to the downregulation of SATB2 observed in colorectal cancer that was associated with metastasis and poor prognosis (Wang et al., 2009). Interestingly, non-OS sarcomas, such as rhabdomyosarcomas, Ewing’s sarcomas and soft tissue sarcomas, were negative for SATB2 immunostaining. Although the diagnosis of OS is fairly straightforward due to the presence of osteoid, the diagnosis of a small percentage (less than 10%) of OS cases can be mis-classified because of the lack of osteoid. Therefore, as a specific marker for OS, SATB2 may assist in difficult diagnoses. More tumour samples (both primary and metastatic OS) will be needed to expand our current data set in order to definitively establish SATB2 as a biomarker for OS and to determine whether SATB2 staining correlates with prognosis or presence of metastases.
I have also demonstrated a role for SATB2 in OS invasion, suggesting a possible role in OS metastasis. In invasion and scratch assays, cells with SATB2 knockdown were less invasive and had a diminished capacity to migrate relative to control cells. These findings indicate that SATB2 promotes cell migration as well as cell invasion of OS cells, thereby suggesting that SATB2 is involved in OS metastasis. Currently, the mechanisms of OS development and metastatic spread are not well understood, although several genes have been implicated, including MMPs, TIMPs and RUNX2. Interestingly, many of these genes that have been previously reported to be associated with OS metastasis have also been linked to SATB2 in OBs. As noted above, SATB2 controls OB differentiation by enhancing the transcriptional activity of RUNX2, a regulator of terminal OB differentiation (Dobreva et al., 2006). In SATB2-deficient OBs (SATB2−/−), MMP3 was downregulated while TIMPs were upregulated compared to SATB2+/− OBs (Dobreva et al., 2006). Interestingly, RUNX2 has been shown to increase the migration of immature OB cells and this was dependent on PI3K/AKT signalling (Fujita et al., 2004), and the PI3K/AKT pathway has been implicated in several metastases-related events, including the activation of the Rho family of GTPases, such as Rac, and proteolytic enzymes, such as MMPs (Jiang and Liu, 2009). Thus, SATB2 may promote cell migration and invasion in concert with RUNX2 by stimulating the activity of Rac in the formation of lamellipodia to allow cell migration and MMPs in the degradation of the ECM to allow cell invasion. Further investigation is therefore needed not only to tease out the unclear roles of some of these genes in OS, but also the signalling pathways downstream of SATB2 in which they act in the metastasis of OS cells. Since tissue invasion and metastasis may be one of the most important hallmarks of cancer (Lazebnik, 2010) and 90% of human cancer deaths result from metastasis (Sporn, 1996), our finding that SATB2 plays a crucial role in OS cell migration and invasion may contribute
further to the understanding of the mechanisms by which cancer cells escape the primary tumour, invade tissues and form new tumours at distant sites.

Since I have shown a role for SATB2 in cell migration, this functional role of SATB2 in OS may be related to its role in OB differentiation. Bone formation requires both migration and differentiation of OBs. Since the first step of bone formation is the migration of OBs and OB progenitors to the site where new bone is required, as is the case for repairing fractures, SATB2 may coordinate OB migration together with RUNX2, which has also been shown to increase the migration of immature OB cells (Fujita et al., 2004). Once at the site of repair, SATB2 and RUNX2 then regulate OB differentiation to complete the formation of new bone. Thus, with its function in cell migration, SATB2 may not solely be a negative factor in OS, but may also play a positive role in bone healing.

Other future areas of study include ongoing analyses of the microarray data in collaboration with Dr. Margaret Pienkowska in Dr. David Malkin’s laboratory. A preliminary analysis showed that many genes are differentially expressed in OS cells lacking SATB2 expression compared to control cells (Table 4.2). Candidate genes identified include those implicated in cell migration [eg. chemokine CXCL10 and roundabout 4 (ROBO4)], cell invasion (eg. MMP16), and cell cycle regulation (eg. E2F5). MMP2 and MMP9 were previously reported to be overexpressed in OS cell lines and these high MMP2 and 9 levels were associated with metastasis (Bjornland et al., 2005). Unexpectedly, our microarray identified another MMP (MMP16) to be upregulated in cells that were shown to have decreased invasive capabilities. Our future experiments will determine whether MMP16 and other candidate genes identified in the microarray to be differentially expressed between OS cells with SATB2 knockdown versus
control cells will be validated and examined for their potential roles in OS invasion and metastasis.

Since *in vitro* migration and invasion assays were used to study the role that SATB2 plays in OS, *in vivo* studies using murine models may be a valuable strategy to more fully evaluate the role of SATB2 in migration and invasion. The experimental metastasis assay in which KHOS-shSATB2 and control cells are injected intravenously into the tail vein or the spontaneous metastasis assay in which these cells are injected directly into the bone to allow for the formation of a primary tumour followed by spontaneous metastases may be useful *in vivo* experiments. In both assays, the mice would be examined for metastases and we would expect that mice that had been injected with KHOS-shSATB2 cells would have fewer metastases than control mice.

In addition, a role for SATB2 in the sensitization of OS to chemotherapy treatment will be investigated since our laboratory has recently shown that SATB2 mediates chemoresistance in HNSCC (Chung et al., 2010). My preliminary studies suggest that, unlike in HNSCC, SATB2 knockdown does not affect chemosensitivity as determined by MTT assays of KHOS cells with and without SATB2 knockdown (data not shown). However, further studies will be undertaken to specifically address whether apoptosis is affected. Furthermore, because SATB1 and SATB2 expression were detected in advanced-stage breast cancer and HNSCC, respectively (Han et al., 2008; Chung et al., 2010), we will examine whether SATB2 expression levels correlate with clinical features of OS tumours (such as response to therapy, survival and metastases). Finally, since I have shown that SATB2 modulates the transcriptional activity and stability of p73 (Chapter 3), we will study whether SATB2 alters p73 function in OS. p53 alterations are present in up to 80% of OS cases (Sandberg and Bridge, 2003) and TAp73 is able to induce apoptosis
independent of p53 (Jost et al., 1997; Kaghad et al., 1997; Ishida et al., 2000). We will therefore investigate whether SATB2 has a survival role in OS by suppressing the pro-apoptotic function of TAp73. Ultimately, this report and future studies may lead to novel drug targets and therapies that may inhibit SATB2 activity, leading to decreased migration and invasion, in the management of OS patients, particularly for those with metastatic disease.
We identified SATB2 by mass spectrometry as a novel p73-binding partner. SATB2 binds to and stabilizes TAp73α, leading to the enhanced transcriptional activity of TAp73α. Conversely, SATB2 does not bind to but decreases TAp73β levels, resulting in inhibition of TAp73β-mediated transcription. Our laboratory has also shown that SATB2 binds to another p53 family member, p63, and SATB2 modulates the activity, but not the stability, of ΔNp63α (Chung et al., 2010). Furthermore, SATB2 discriminates p73 at the C-terminus (binds to p73α but not p73β variants), whereas SATB2 is indiscriminate at the N-terminus (binds to both TAp73α and ΔNp73α isoforms). Interestingly, in contrast to p73, SATB2 binding is not specific for p63 C-terminal isoforms (Chung et al., 2010 and data not shown). Thus, SATB2 has been shown to be both a co-activator and co-repressor, and perhaps its role as a p73/p63-binding partner depends on the specific isoform expression profile in a given cell. Moreover, our findings reveal a role for SATB2 as a regulator of protein stability that is specific to p73.

Potential role of p73-SATB2 interaction in the developing nervous system

Both p73 and SATB2 have been independently implicated in neurodevelopment. Studies of p73-null mice (p73<sup>−/−</sup>), which lack all p73 isoforms, revealed that ΔNp73β is the predominant isoform of p73 that is expressed in the developing murine nervous system, and that loss of this anti-apoptotic p73 isoform led to increased apoptosis of peripheral sympathetic and cortical neurons (Pozniak et al., 2000; Pozniak et al., 2002). p53 and TAp63γ can both induce apoptosis in
developing neurons (Jacobs et al., 2005). Since, p53 requires TAp63 to induce apoptosis but TAp63γ can induce apoptosis in the absence of p53, TAp63γ is the dominant cell death protein in developing neurons (Jacobs et al., 2005). Therefore, by opposing the apoptotic function of p53 and TAp63, ΔNp73 acts as a pro-survival protein in developing neurons (Pozniak et al., 2000; Jacobs et al., 2005). SATB2 is expressed in post-mitotic, differentiating cortical neurons, and its expression is higher in differentiating neurons and lower in mature neurons (Britanova et al., 2005; Szemes et al., 2006). Moreover, SATB2 is essential in the development of callosal projection neurons, allowing these neurons to extend across the corpus callosum to the other cerebral hemisphere (Alcamo et al., 2008; Britanova et al., 2008). Thus, SATB2 and p73 have separate roles in developing neurons.

In HNSCC, two predominant p53 family variants—TAp73β and ΔNp63α—are present and our laboratory has shown that SATB2 tips the balance of the TA:ΔN ratio in favour of survival by enhancing the ΔNp63-mediated repression of pro-apoptotic genes in HNSCC (Chung et al., 2010). Similar to HNSCC, two predominant p53 family isoforms—TAp63γ and ΔNp73β—have been detected in developing neurons. Since both SATB2 and p73 are expressed in developing neurons and I have already demonstrated a link between SATB2 and p73 (Chapter 3), perhaps a role for their interaction may be discerned in this system. During neural development, neuronal death naturally occurs mainly after the post-mitotic differentiated neurons have extended axons and made connections. These neurons then compete for nerve growth factor (NGF) released by target tissues and those that do not receive enough undergo apoptosis (Jacobs et al., 2006). It is conceivable that, in developing neurons, SATB2 regulates the predominant p73 isoform, ΔNp73β. I have shown that SATB2 is able to modulate ΔNp73β levels (Figure 3.9B). Since I have observed that SATB2 co-expression leads to decreased
TAp73\(\beta\) levels (Figure 3.9) and inhibition of its transcriptional activity (Figure 3.7, lane 6), SATB2 may modify the pro-survival function of \(\Delta Np73\beta\). Therefore, SATB2 may contribute to the regulatory balance of the TA:\(\Delta N\) ratio of the p53 family members by repressing the expression levels and the inhibitory effect of \(\Delta Np73\beta\), which supports the pro-apoptotic function of TAp63\(\gamma\) in developing neurons (Figure 5.1). Hence, SATB2 may indirectly promote TAp63\(\gamma\)-dependent cell death for neurons that are unable to obtain enough NGF for survival. This model is further supported by the fact that SATB2 expression was higher in differentiating neurons and lower in mature neurons (Britanova et al., 2005). Hence, once connections have been established and the neurons lacking these connections have undergone apoptosis, SATB2 expression potentially decreases. Reduced SATB2 expression would result in increased \(\Delta Np73\beta\) levels, which would lead to suppression of TAp63\(\gamma\) and the subsequent survival of these established neurons. In this way, SATB2 may alternate between the pro-apoptotic and pro-survival outcomes mediated through TAp63\(\gamma\) and \(\Delta Np73\beta\), respectively. A role for the interaction between p73 and SATB2 in neurodevelopment should therefore be investigated in future studies, as well as a direct effect of SATB2 on TAp63\(\gamma\) since our laboratory has already demonstrated a link between SATB2 and \(\Delta Np63\alpha\) in HNSCC (Chung et al., 2010).
Figure 5.1. Model for SATB2 regulation of p53 family proteins in HNSCC and neurons. The left panel shows the regulation of HNSCC survival by SATB2. SATB2 expression leads to decreased levels of TAp73β and also enhances ΔNp63α-mediated inhibition of TAp73β activity. The right panel shows the proposed regulation of developing neurons by SATB2. SATB2 expression leads to decreased levels of ΔNp73β, which alleviates the inhibition of TAp63γ, thereby promoting neuronal cell death.
In inhibiting protein-protein interactions of p73 as an anti-cancer strategy

In addition to binding to p73α (Chapter 3), our laboratory recently showed that SATB2 also binds to ΔNp63α and this interaction plays a pro-survival role in HNSCC (Chung et al., 2010). SATB2 is expressed in advanced-stage HNSCC and promotes chemoresistance by enhancing ΔNp63α binding to p53-target gene promoters, resulting in the inhibition of TAp73β-mediated induction of pro-apoptotic target genes (Rocco et al., 2006; Chung et al., 2010). I have also shown that, although SATB2 does not bind to TAp73β, SATB2 expression leads to decreased levels of TAp73β in HNSCC cells (Figure 3.9D). Therefore, SATB2 mediates survival of HNSCC cells in two ways—by enhancing the inhibitory activity of ΔNp63α, and by decreasing TAp73β levels (Figure 5.1, left panel). The net effect is reduced induction of apoptosis of HNSCC cells due to the diminished levels of TAp73β, as well as the inhibition of any TAp73β in these cells by ΔNp63α. Thus, targeting SATB2 as an anti-cancer strategy to block its pro-survival function via its interaction with ΔNp63α, at least in HNSCC, would result in increased apoptosis mediated by TAp73β, especially in conjunction with chemotherapy treatment.

The p53 pathway has been a focus of intense study to develop anti-cancer agents in recent years. Since MDM2 binds to and mediates the proteasomal degradation of p53 (Haupt et al., 1997; Honda et al., 1997; Kubbutat et al., 1997) and its amplification is present in about 10% of all human cancers (Toledo and Wahl, 2006), including OS, one of the most promising new targeting strategies for cancer therapy takes advantage of blocking this inhibitory effect of MDM2 on p53. Nutlin, the first small-molecule MDM2 inhibitor, has been shown to be effective in re-activating p53 by occupying the p53-binding site on MDM2 (Vassilev et al., 2004). However, a possible negative consequence of targeting the MDM2-p53 interaction
results from the negative feedback loop that is generated by the inhibition of MDM2, which leads to accumulation of p53 and subsequent induction of MDM2 by p53 (Barak et al., 1993). Moreover, p53 is mutated in many tumours, thereby rendering this strategy less effective. Furthermore, activating p53 “gain of function” mutants that inhibit the pro-apoptotic abilities of p73 (Di Como et al., 1999; Marin et al., 2000; Strano et al., 2000; Gaiddon et al., 2001) would also be potentially unfavourable. Therefore, targeting other aspects of the p53 pathway or focusing on the p73 pathway, especially since p73 is able to induce apoptosis independent of p53 (Jost et al., 1997; Kaghad et al., 1997; Ishida et al., 2000), may be alternatives for anti-cancer strategies given some of the limitations of the MDM2-p53 axis.

Since the majority of human cancers exhibit either mutation or inactivation of p53 (Vogelstein et al., 2000), targeting protein-protein interactions of p73 may be a more viable option in the fight against cancer. For example, MDM2 binding to p73 leads to repression of the transcriptional activity of p73 (Balint et al., 1999; Dobbelstein et al., 1999; Zeng et al., 1999), but Nutlin is effective in disrupting the MDM2-TAp73 interaction by binding to MDM2, thereby activating TAp73-mediated apoptosis in some cancer cells (Lau et al., 2008). TAp73, therefore, is able to induce apoptosis independent of p53, which is important in sensitizing tumours with mutated or inactivated p53 to chemotherapy (Irwin et al., 2003). Since Nutlin was originally discovered as an antagonist to the MDM2-p53 interaction but is also able to inhibit MDM2 binding to TAp73, additional inhibitors targeting the MDM2-p53 axis should be examined for the ability to block the MDM2-TAp73 interaction.

Another inhibitory protein-protein interaction for p73 that may be worth considering as a therapeutic target involves the p53 “gain of function” mutants that bind to and inhibit the pro-apoptotic activity of TAp73 (Di Como et al., 1999; Marin et al., 2000; Strano et al., 2000;
Gaiddon et al., 2001). I have already shown that SATB2 can bind to TAp73α and enhance its transcriptional activity (Chapter 3). Perhaps by binding, SATB2 may prevent mutant p53 or other inhibitory proteins from binding to TAp73α. The net effect of SATB2 would depend on the C-terminal variant of p73 that is being repressed by mutant p53. For example, if mutant p53 bound to TAp73α, SATB2 may be able to displace mutant p53 by binding to and enhancing the pro-apoptotic activity of TAp73α. In cancers, such as neuroblastoma, with high levels of ΔNp73α, SATB2 may have pro-survival activities (similar to SATB2 and ΔNp63α in HNSCC) and in this system, inhibiting SATB2 may lead to cell death. Further investigation into whether SATB2 functions in this capacity should be investigated for its potential as an anti-cancer strategy. Therefore, SATB2 may have opposing activities in cells, depending on the specific p53 family isoforms expressed in a certain cancer type.

### Role of SATB2 in OS and the potential involvement of the p53 family

We have shown that SATB2 is overexpressed in OS and demonstrated a role for SATB2 in OS migration and invasion, thereby suggesting a possible role in OS metastasis (Chapter 4). Studies furthering our understanding of the role of SATB2 in OS include ongoing microarray analyses to discover novel SATB2 target genes and/or metastasis-associated gene candidates that are differentially expressed in OS cells with SATB2 knockdown versus control. A preliminary analysis is shown in Table 4.2 and we are currently validating the results with qRT-PCR. Since the SATB2 family member, SATB1, has been reported to upregulate metastasis-associated genes in breast cancer cells (Han et al., 2008) and I have shown that SATB2 has a role in OS invasion, we hypothesize that SATB2 may similarly alter the expression profile of genes associated with metastasis. These candidate genes will also be examined to determine whether they overlap with
any known targets of the p53 family, RUNX2, or the SATB family in non-OS cells. Specific candidate genes identified in the microarray that we have prioritized to validate include chemokines and matrix metalloproteinases. For example, the chemokine CXCL10, which was upregulated in KHOS-shSATB2-1 cells compared to control OS cells has both anti- and pro-metastatic roles. CXCL10 is an inhibitor of angiogenesis and has also been shown to promote migration and invasion in melanoma and multiple myeloma cells (Singh et al., 2007). The expression of MMPs and inhibitors of MMPs, such as TIMPs, have been studied in OS tumours, although the upstream pathways that regulate MMP expression in OS have not been well described. In my studies, MMP16 was unexpectedly upregulated in less invasive cells (KHOS-shSATB2). If this finding is validated, we will specifically address whether MMP16 has a different role than other MMPs in OS. Furthermore, the levels of MMP2 and MMP9 were higher in OS cell lines that were more invasive (Bjornland et al., 2005). Interestingly, MMP9 has been reported to be a RUNX2 target gene (Pratap et al., 2005) and, in OS cell lines, MMP9 overexpression was associated with metastasis (Bjornland et al., 2005). Since SATB2 has been linked with the MMP family in OBs (Dobreva et al., 2006) and RUNX2 can regulate MMP expression (Pratap et al., 2005), we will investigate whether SATB2 and RUNX2 are co-regulators, and together promote cell invasion and metastasis in OS.

Alterations in the p53 pathway, including inactivation or mutation in up to 80% of sporadic tumours, have been reported in OS (Sandberg and Bridge, 2003). Moreover, deletion of p53 specifically in OBs was sufficient for mice to develop OS (Walkley et al., 2008; Berman et al., 2008). Since, like p73β, SATB2 co-expression results in decreased p53 levels (Figure 3.9C), p53 may be alternatively inactivated in OS by the elevated levels of SATB2 expression. Interestingly, in a comparison of various OS cell lines (Figure 4.1), I observed that SATB2 levels
were highest in Saos-2 cells (lane 6), which are \( p53^{-/-} \), and lowest in U2OS cells (lane 5), which express wild-type p53. This observation suggests that, at least in a subset of OS cell lines, SATB2 expression may correlate with p53 status. Thus, future studies to knockdown or overexpress p53 may indicate whether p53 directly influences SATB2 levels. Although preliminary studies into the levels of p73 in our OS cell lines have not demonstrated an obvious correlation between p73 and SATB2 levels, the fact that SATB2 is able to modulate p73 levels in an opposing manner dependent on the C-terminal isoform expressed warrants further investigation (see below). One group has reported on p63 and p73 expression in OS—decreased \( TAp63 \) and \( TAp73 \) mRNA expression were detected in 67% and 50%, respectively, of OS samples when compared to normal bone tissue (Park et al., 2004). In addition, p63 plays a role in bone development as evident by the skeletal defects, such as limb and craniofacial abnormalities, present in \( p63^{-/-} \) mice (Mills et al., 1999; Yang et al., 1999). Thus, potential roles for all three members of the p53 family have been reported in bone development and/or OS.

Since we have shown elevated levels of SATB2 expression in OS (Chapter 4) and I have also demonstrated a link between SATB2 and p73 (Chapter 3), it is tempting to speculate that the interaction between SATB2 and p73 may play a role in OS and metastasis. Although I have not formally tested whether p73 mediates the decreased migration and invasion seen in KHOS-shSATB2 cells, preliminary IBs of the various OS cell lines with differential SATB2 protein levels did not show any obvious correlation between p73 and SATB2 (data not shown). However, this potential relationship may be addressed by examining the p73 protein levels in KHOS-shSATB2 cells compared to control to determine whether there are any differences. p73 may also be expressed in these cells and then used in the migration and invasion assays to
examine whether p73 alters the ability of these cells to migrate or invade in the absence or presence of SATB2. In addition, low levels of TAp73β detected in OS tumours may result from elevated expression levels of SATB2, which potentially destabilizes TAp73β (Figure 3.10). In TAp73-deficient mice, loss of TAp73 was associated with increased genomic instability in lung tissue, thereby resulting in cancers developing primarily in the lung (Tomasini et al., 2008). Interestingly, the lung is the primary site of metastases in OS. Since high levels of SATB2 may lead to destabilization and therefore loss of TAp73β expression and activity, the combined effect of increased SATB2 and decreased TAp73β levels may result in increased migration and invasion in OS. Thus, if we target SATB2 in this case, reduced levels of SATB2 may lead to a release of its inhibitory effect on TAp73β, thereby allowing TAp73β expression levels to rise and activation of pro-apoptotic gene promoters, as well as potential inhibition of metastatic spread. Moreover, ΔNp73 overexpression is common in many types of cancers (Buhlmann and Putzer, 2008). In tumours with high ΔNp73α expression, it would not only be stabilized by SATB2, but it would also inhibit TAp73-mediated apoptosis (Figure 3.10). Again, targeting SATB2 would be beneficial since reducing its expression levels would lead to decreased levels of ΔNp73α but increased TAp73β. Our laboratory has already shown that reduced levels of SATB2 expression can sensitize HNSCC cells to apoptosis induced by chemotherapeutic treatment (Chung et al., 2010). If SATB2 is targeted in combination with chemotherapy, patients with OS, especially those with mutated or inactivated p53, would benefit from enhanced chemosensitivity due to the activity of TAp73 (Irwin et al., 2003). Therefore, it is important that future studies differentiate between the TA and ΔN isoforms of p73, as well as the C-terminal variants, in order to more effectively develop anti-cancer agents that target the protein-protein interactions of p73, such as those involving SATB2.
In summary, I have demonstrated that SATB2 is a novel p73-interacting protein that differentially regulates the C-terminal isoforms, α and β, of p73. We have also shown that SATB2 is overexpressed in OS, both at the mRNA and protein level, and may be a potential biomarker distinguishing OS from other sarcomas, thereby aiding in the diagnosis of this cancer. I have additionally demonstrated that SATB2 promotes cell migration and invasion of OS cells since loss of SATB2 inhibits these functions. I have proposed possible functions of this interaction in both neurodevelopment and as an anti-cancer strategy, targeting both SATB2 itself and other protein-protein interactions of p73. Hence, further studies of the relationship between SATB2 and p73 should be investigated for the role of SATB2 as a novel regulator of p73 stability.
References


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References


